THE ROLE OF HYDROGEN SULFIDE IN NORMAL AND ISCHEMIC HEART

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Summary

Ischemic heart disease is the leading cause of death in the western society and a major health problem in developing countries. In the current study, the role of hydrogen sulfide (H_2S) in the cardioprotection against ischemic heart injury was investigated.

Firstly, the role of H_2S in excitation-contraction coupling in cardiomyocytes was studied. H_2S was shown to negatively modulate the β-adrenergic system, which is over-stimulated during ischemia/reperfusion, via inhibiting adenyly cyclase activity. This inhibition resulted in reduced cAMP production, and thus may prevent calcium overload-induced ventricular arrhythmias. Further experiments were conducted to confirm the cardioprotective effects of H_2S in isolated rat heart and cardiomyocytes. Endogenous H_2S production in heart was found to be suppressed in cardiomyocytes subjected to ischemia. Preconditioning or postconditioning the hearts with several episodes of brief ischemia significantly restored the H_2S production in the heart accompanied by improved heart contractile function during reperfusion. Inhibition of H_2S synthesis partially blocked the cardioprotective effect of both pre- and post-conditioning, indicating that endogenous H_2S may, at least in part, mediate the protection given rise by these two maneuvers. The present study also demonstrated that NaHS, an H_2S donor, was an effective pharmacological pre- and post-conditioning agent to ameliorate the cardiac injury induced by ischemia/reperfusion (I/R) in terms of cells death, cell morphology, intracellular calcium handling, cellular and heart contractile function, infarction size, and arrhythmias.

The interaction between H_2S and nitric oxide (NO), two important gasotransmitters, was also studied in this thesis. Mixture of NaHS with different NO

donors and L-arginine, a main substrate for NO synthase to generate NO, exerted completely opposite effects on myocytes contractile function and calcium cycling, suggesting that a novel reaction product of H_2S +NO, may be formed. Additional experiments demonstrated that this novel compound may be nitroxyl since this novel substance possesses several properties very similar to that of nitroxyl, like producing positive inotropic effect via cAMP/PKA, cGMP/PKG independent pathways, in which their effects were sensitive to thiols.

In conclusion, H_2S may negatively modulate the β-adrenergic system which translates H_2S into a good cardioprotective agent to protect the heart from ischemia/reperfusion injury, when the β-adrenergic receptor is over-stimulated. In addition, the present study also demonstrated that H_2S may sophisticatedly regulate excitation-contraction coupling in the heart by modulating intracellular calcium in a totally different manner in the presence of NO, suggesting the formation of a novel compound, which potentially plays a significant role during certain conditions like inflammation, when both gasotransmitters are highly produced.

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Symbols	Full name
3MST	3-mercaptopyruvate sulphurtransferase
8B-cAMP	8-bromo-cyclic-adenosine monophospate
AC	Adenylyl cyclase
ANOVA	One-way analysis of variance
APD	Action potential duration
AS	Angeli's salt
ATP	Adenosine triphosphate
AV	Atrioventricular
BayK	Bay K-8644
BCA	β-cyano-L-alanine
BSM	Bisindolymaleimide
Ca^{2+}	Calcium
CABG	Coronary artery bypass grafting
CamKII	Ca^{2+} /Calmodulin-Dependent Protein Kinase II
cAMP	Cyclic-adenosine monophospate
CBS	Cystathionine β-synthase
CICR	Ca^{2+}-induced Ca^{2+} release
CNS	Central nervous system
CO	Carbon monoxide
CSE	Cystathionine-γ-lyase
CVD	Cardiovascular disease

DAD	Delayed afterdepolorizations
DEA/NO	Diethylamine NONOate sodium salt hydrate
ECG	Electrocardiogram
EI	Electrically-induced
Emax	Maximal effect
eNOS	Endothelium nitric oxide synthase
ERK1/2	Extracellular signal regulated kinase 1/2
GSH	Glutathione
H_2S	Hydrogen sulfide
HNO	Nitroxyl
IL-1	Interleukin 1
iNOS	Inducible nitric oxide synthase
IP	Ischemic preconditioning
IPostC	Ischemic postconditioning
ISO	Isoproterenol
JNK	Jun N-terminal kinase
K_{ATP}	ATP-sensitive-Potassium
LAD	Left anterior descending coronary artery
L-arg	L-arginine
L-cys	L-cysteine
LVDP	Left ventricular developed pressure
LVeDP	Left ventricular end diastolic pressure
MAPK	Mitogen-activated protein kinase

MI	Myocardial infarction
mitoK$_{ATP}$	Mitochondrial ATP-sensitive potassium
NAC	N-acetyl-cysteine
NCX	Sodium-calcium exchanger
NMDA	N-methyl-D-aspartic acid
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
PAG	DL-propargylglycine
PCI	Percutaneous coronary intervention
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein Kinase G
PLB	Phosopholamban
PLC	Phospholipase C
PLP	Pyridoxal 5'-phosphate
PVC	Premature ventricular contraction
ROS	Reactive oxygen species
Rp-cAMP	Rp-Adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt hydrate
Rp-cGMP	8-(4-Chlorophenylthio)-guanosine 3′,5′-cyclic monophosphorothioate, Rp Isomer triethylammonium salt
RyR	Ryanodine receptor

SA	Sinoatrial
sarcK_{ATP}	Sarcolemmal ATP-sensative potassium
SERCA	Sarcoplasmic/Endoplasmic reticulum calcium ATPase
SMCs	Smooth muscle cells
SNP	Sodium nitroprusside dihydrate
SP	NaHS preconditioning
SR	Sarcoplasmic reticulum
t50	Half-decay time
t90	90%-decay time
Thap	Thapsigargin
TNFα	Tumor necrosis factor-α
VF	Ventricular fibrillation
VP	Vehicle preconditioning
VPostC	Vehicle postconditioning
VT	Ventricular tachycardia
β-AR	β-adrenergic receptor
$[Ca^{2+}]_i$	Intracellular calcium
+dP/dt	Contractility, maximum gradient during systoles
-dP/dt	Compliance, minimum gradient during diastoles
±dL/dt	Maximum velocity of cell shortening or relaxing

Chapter 1 Introduction

1.1. General Overview

The cardiovascular system consists of the heart and blood vessels which provides the tissues/organs of the body with a continuous supply of oxygen, nutrients, and waste removal. The heart is the first organ formed during embryonic development and is responsible for circulating approximately 7200 liters of blood per day throughout the vasculature of a human adult. The mammalian heart is comprised of four chambers, two atria and two ventricles operating in a series of electrical and mechanical events that control blood flow into and out of the heart. A region of the heart called the sinoatrial (SA) node is capable of producing and discharging an action potential and sending the impulse across the atria to cause both left and right atria to contract in unison. The impulses then pass to the atrioventricular (AV) node, and the signal is further conducted by a specialized muscle fiber, Purkinje fibers, to the apex of the heart and throughout the ventricular walls. The impulses generated during the heart cycle produce small electrical currents, which are conducted through body fluids to the skin, where they can be detected by electrodes and recorded as an electrocardiogram (ECG). Over the past 100 years, contractile functions of the heart have been extensively studied, and we now understand the basic mechanisms of heart contraction and relaxation.

1.2. Excitation-contraction coupling

In adult mammalian hearts, excitation-contraction coupling, the key determinant of cardiac function, is the process from electrical excitation to contraction of the myocyte (Bers, 2002; Fabiato and Fabiato, 1977). During a cardiac action potential, upon the

depolarization of sarcolemma, Ca^{2+} enters the cell through L-type Ca^{2+} channel, as an inward Ca^{2+} current (I_{Ca}), which activates the sarcoplasmic reticulum (SR) Ca^{2+} release channel, ryanodine receptor (RyR2), triggering Ca^{2+} release from the SR. This process is termed as Ca^{2+}-induced Ca^{2+} release (Bers, 2002). The combination of Ca^{2+} influx and release raises the free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) from 150nM to 1μM, allowing Ca^{2+} to bind to the myofilament protein troponin C, which then initiates contraction (Bers, 2002). For relaxation to occur, Ca^{2+} must be removed from the cytosol, allowing Ca^{2+} to dissociate from troponin C (Solaro and Rarick, 1998). Four separate Ca^{2+} handling systems participate in the removal of Ca^{2+}: 1) SR Ca^{2+}-ATPase (SERCA2a), 2) sarcolemmal Na^{+}-Ca^{2+} exchanger (NCX), 3) sarcolemmal Ca^{2+}-ATPase and 4) mitochondrial Ca^{2+} uniport (Bassani et al., 1994; Bers, 2002; Lederer et al., 1990; Shannon and Bers, 2004). Although the contribution of NCX and SERCA2a to Ca^{2+} decline is species-dependent, the sarcolemmal Ca^{2+}-ATPase and mitochondrial Ca^{2+} uniport generally play a minor role in the Ca^{2+} decline (~ 1-2% of the Ca^{2+}) during relaxation (Bassani et al., 1992; Bers et al., 1993).

1.2.1. Intracellular calcium cycling in adult mammalian hearts

In adult mammalian hearts, SR Ca^{2+} cycling plays a key role in the intracellular Ca^{2+} homeostasis and the regulation of cardiac function (Fabiato and Fabiato, 1977; Lederer et al., 1990). The SR Ca^{2+} release during each cardiac cycle is the determinant of the force generated and the SERCA2a Ca uptake plays a central role in controlling the SR Ca^{2+} load and cardiac relaxation (Baker et al., 1998; Luo et al., 1994). However, the trans-sarcolemma Ca^{2+} cycling systems, i.e. L-type Ca^{2+} channel and NCX, are also important for the regulation of intracellular Ca^{2+} cycling and excitation-contraction

coupling. Specifically, to maintain intracellular Ca^{2+} homeostasis and normal cardiac function, the Ca^{2+} efflux via NCX must be matched by the Ca^{2+} influx from L-type Ca^{2+} channel (Haddock et al., 1998). The Ca^{2+} release from SR must be equal to SERCA2a Ca^{2+} re-uptake during each steady-state heartbeat (Shannon and Bers, 2004). Thus, the regulation of each system is critical for normal cardiac contractile function on a beat-to-beat basis.

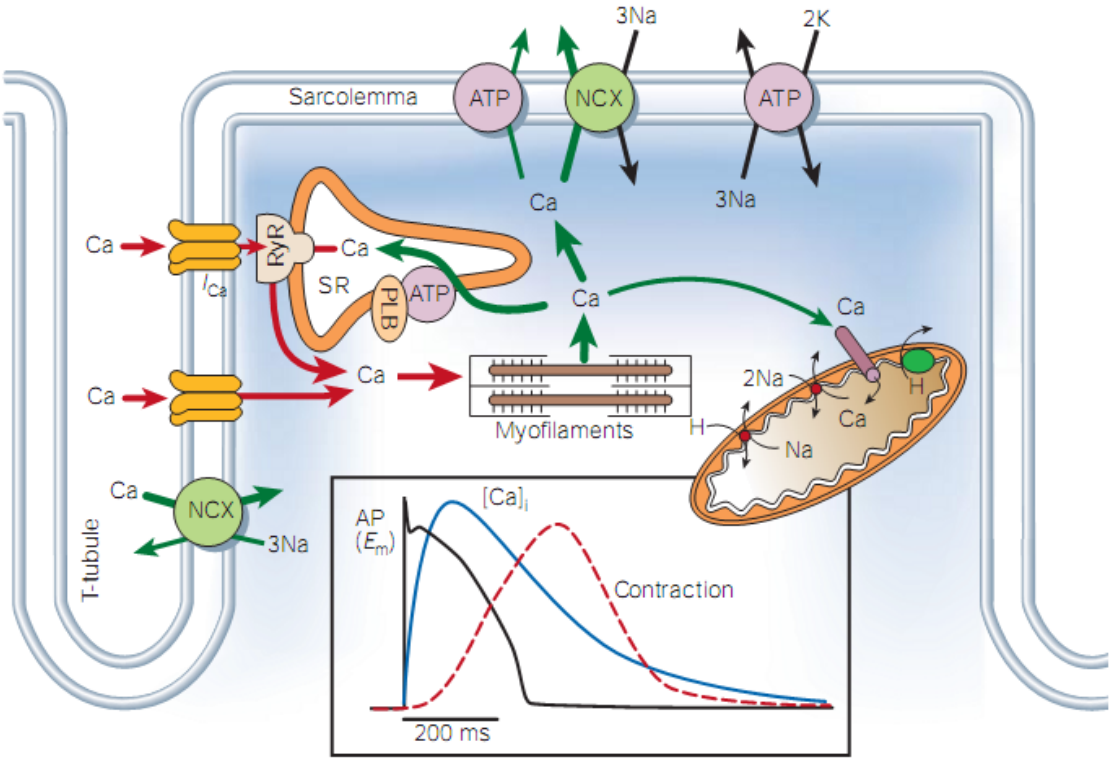


Figure 1-1 Calcium transport in ventricular myocytes. Inset shows the time course of an action potential, calcium transient and contraction measured in a rabbit ventricular myocytes at 37˚C. NCX, Na+/Ca2+ exchanger; ATP, ATPase; PLB, phospholamban; SR, sarcoplasmic reticulum.
This figure is obtained from Bers (2002) (Bers, 2002)

1.2.1.1. Voltage-dependent L-type Ca^{2+} channel

The core cardiac L-type voltage-dependent Ca^{2+} channel is heterotetrameric polypeptide complex composed of α1c subunit, the transmembrane α2/δ subunit and the cytoplasmic β subunit located within transverse tubule network. A propragating action potential down the transverse-tubules activate the voltage-sensitive α1 subunit Ca^{2+} pore facilitating extracellular Ca^{2+} entry, whereas α2/δ and β subunits are auxillary components in this process (Gurnett and Campbell, 1996). The L-type Ca^{2+} channel is the link between electrical excitation and mechanical contraction in the cardiomyocyte by initiating the first step in Ca^{2+} mobilization. In cardiomyocytes, this channel is the main port for Ca^{2+} entry controlling intracellular Ca^{2+} concentration, ultimately determining the strength of contraction. This important role explains the convergence of multiple signalling cascades regulating the activity of the L-type Ca^{2+} channel protein. Single channel and whole cell patch-clamp analysis demonstrated Ca^{2+} inward amplitude can be increased by several phosphorylating kinases: PKA, PKC cGMP-dependent kinase, and calmodulin kinase II (Mori et al., 1996; Muth et al., 1999). Enhancing Ca^{2+} entry elicits an increasing SR Ca^{2+} release, generating a graded contractile response in cardiomyocytes. Increase of Ca^{2+} entry augments existing cytosolic and SR Ca^{2+} stores, inducing stronger contraction within the sarcomeric machinery in subsequent rounds of excitation-contraction coupling (Houser et al., 2000). SR Ca^{2+} release increases regional Ca^{2+} concentration surrounding the L-type Ca^{2+} channel, which in turn induces Ca^{2+}-dependent inactivation of the L-type Ca^{2+} channel by closing the gating mechanism within the α1c-subunit. Slower or reduced SR Ca^{2+} release decreases the rate of inactivation of the channel.

1.2.1.2. Ryanodine receptor

The ryanodine receptor, or Ca^{2+} release channel, was initially characterized as a ~565 kDa protein (Otsu et al., 1990) that forms homotetramers (~2.2 MDa) on the junctional sarcoplasmic membrane. Each subunit contains a large cytosolic domain and a smaller intra-membrane domain surrounding a Ca^{2+}-specific central pore perforating the sarcoplasmic reticulum membrane. Mammalian tissues express three receptor isoforms: RyR1, skeletal muscle; RyR2, cardiac muscle; and RyR3, brain tissue (Hamilton and Serysheva, 2009). Cardiac RyR2 is functionally unique from skeletal RyR1 in that SR Ca^{2+} release is induced by Ca^{2+} and is not mechanically coupled to L-type Ca^{2+} channels as in skeletal muscle (Fabiato and Fabiato, 1978). In this context, cardiac SR Ca^{2+} release is a graded response depends on the amount of calcium influx through L-type Ca^{2+} channel, which is critical for cardiac reserve and variable force generation in cardiomyocytes. Recent reports documented that L-type Ca^{2+} channels and RyR2 receptors form functional clusters in the space between the sarcolemmal and SR membranes in cardiac cells (MacLennan et al., 2002). Indeed, functional activation of single or multiple groups of these clusters give rise to a "Ca^{2+} spark" which can be visualized by Ca^{2+} sensitive dyes in isolated cardiomyocytes (Cheng et al., 1993).

RyR2 function is regulated by activating agonists such as low concentrations of ryanodine and calmodulin, caffeine, and ATP in the presence of Ca^{2+} (Ikemoto et al., 1995; Meissner and Henderson, 1987; Rousseau and Meissner, 1989; Smith et al., 1988; Tripathy et al., 1995), causing conformational rotation of the tertiary complex to the open position and allowing Ca^{2+} flow from the SR. Additionally, high luminal SR Ca^{2+} concentration enhances the open probability of the RyR2, whereas low Ca^{2+}

concentration tends to reduce this activity (Fill and Copello, 2002). Recent reports show that hyperphosphorylation of the RyR2 channel and associated proteins may affect SR Ca^{2+} loading by increasing the open probability of the channel (Marx et al., 2000), however, this concept is not universally accepted (Jiang et al., 2002; Li et al., 2002) , which suggests SR Ca^{2+} content is primarily dependent on the activity of SERCA protein.

1.2.1.3. Sarcoplasmic reticulum Ca^{2+} ATPase

SERCA2a is the primary Ca^{2+} transporter in the heart, utilizing the energy from ATP hydrolysis to relocate Ca^{2+} ions against a ~1000-fold concentration gradient into the SR lumen (Hasselbach and Oetliker, 1983). Active Ca^{2+} transport is accomplished through small energetically-favorable steps dependent on cytosolic Ca^{2+} and ATP levels (Katz, 2001). Kinetically, SERCA2 activity is directly regulated by phosopholamban (Luo et al., 1994). Dephosphorylated phospholamban binds to SERCA2 in a monomeric conformation, inhibits rate-limiting steps in enzymatic reaction kinetics (Katz, 2001) for Ca^{2+} reuptake, slows down SR Ca^{2+} loading and affects sarcomeric relaxation. Phosphorylation of phospholamban by PKA (Tada et al., 1983) and CamKII (Kranias et al., 1980) turns phospholamban into a pentameric form, thereby releasing SERCA2 inhibition and increasing SR Ca^{2+} uptake and ATPase activity (Tada et al., 1982). Cardiac-specific ablation of phospholamban, which increases SR Ca^{2+} loading, is accompanied by accelerated muscle relaxation in knockout mice. (Bluhm et al., 2000) Conversely, overexpression of phospholamban significantly inhibits SERCA activity, and affects Ca^{2+} cycling which leads to cardiomyopathy (Dash et al., 2001) in transgenic mice.

Dependence on SERCA2 for cytosolic Ca^{2+} uptake varies among species. Mice and rats predominantly rely on SERCA2 (~90%) for diastolic relaxation. Dependency shifts to ~70% in larger animals including humans (Bers, 2002), delegating the remainder of Ca^{2+} uptake to the NCX (~28%) and slow sarcolemmal Ca^{2+} ATPases (~1%) (Bers, 2002). Regardless of species, SERCA2 remains as the predominating protein responsible for SR Ca^{2+} loading and relaxation in cardiomyocytes.

1.2.1.4. Na^{+}-Ca^{2+} Exchanger

The NCX catalyzes exchange of three Na^{+} ions (influx) for one Ca^{2+} ion (efflux) creating an electrogenic gradient across the plasma membrane (Shigekawa and Iwamoto, 2001). Recent studies demonstrated that the mature cardiac NCX1 isoform (~120 kDa) can functionally operate in two modes, forward and reverse (Bers, 2002). Depending on the electrical activity of the cardiomyocyte, Ca^{2+} is extruded (forward mode) or entered (reverse mode) the myocyte. Although the capacity of the reverse mode on excitation-contraction coupling remains controversial (Bers, 2002), it is widely accepted that forward mode plays a supplementary role in diastolic relaxation. Overexpression of NCX1 protein in transgenic mice was shown to enhance Ca^{2+} transient recovery and myocyte contractility (Yao et al., 1998). In contrast, adenovirally infected rabbit myocytes overexpressing NCX showed abnormal contractility and Ca^{2+} handling, illustrating the disparity between human and rodent dependency on this molecule (Ranu et al., 2002).

Unlike SERCA2, L-type Ca^{2+} channel, and RyR2 which are regionally localized, NCX is spatially arranged throughout the sarcolemmal membrane and intercalated disks (Shigekawa and Iwamoto, 2001) returning Ca^{2+} to the extracellular space and

counterbalancing Ca^{2+} entry via the L-type Ca^{2+} channel. NCX activity is affected by intracelluar and extracellular Na+ and Ca^{2+} concentrations. Interestingly, removing extracellular Na^{+} and Ca^{2+} ions in buffer solutions surrounding isolated cardiomyocytes inhibits forward mode exchanger activity, preventing Ca^{2+} extrusion during diastole (Yao et al., 1998). Receptor mediated stimulation by PKC and possibly PKA signaling cascades result in activation of NCX which in turn enhances forward mode Ca^{2+} extrusion. However, the precise mechanisms by which these changes occur on NCX protein remain controversial (Shigekawa and Iwamoto, 2001).

1.2.2. β-adrenergic signaling

1.2.2.1. Effect of β-adrenergic signaling on Ca^{2+} cycling and cardiac function

The sympathetic nervous system is characteristically responsible for the "flight" or stress response program in mammals. With regards to the heart, the β-adrenergic signalling pathway is the primary mechanism that transiently increases cardiac output. The β-adrenergic receptors are transducers which link hormone-mediated chemical signals to the mechanical event of augmented myocardial contraction. Of the three known β-receptor isoforms (β1, β2 and β3), β1- and β2-receptors primarily transduce neurohormonal input into the myocyte, with β1-receptor as the major subtype (~70-80%) (Dorian, 2005). In cellular level, the classic route of contractile function stimulation is the result of the activation of adenylyl cyclase (AC) catalytic activity by the β-adrenoceptor-coupled stimulatory G protein, which leads to increased intracellular cAMP level. This in turn stimulates protein kinase A (PKA) which then mediates phosphorylation of L-type Ca^{2+} channels leading to Ca^{2+} influx to the intracellular compartment. Ca^{2+} entry triggers Ca^{2+} release from sarcoplasmic reticulum and the

elevated free intracellular Ca^{2+} ($[Ca^{2+}]_i$) bind to the myofilament protein troponin C, which then switch on the contractile machinery (Bers, 2002). In addition, activation of PKA also directly phosphorylates RyR2 and PLB. The former increases the open probability of RyR2 in SR (Takasago et al., 1989), whereas the latter stimulates SR-Ca^{2+} uptake (Simmerman and Jones, 1998). Troponin I is an inhibitory protein associated with troponin C and T complex on tropomyosin (Bers, 2002). Enhanced phosphorylation of troponin I decreases troponin C affinity for Ca^{2+}, which results in faster relaxation of the sarcomere (Bers, 2002). Collectively, these changes increase the Ca^{2+} transient amplitude, decrease diastolic relaxation time, and increase force generation of the myocyte, ultimately increasing cardiac output.

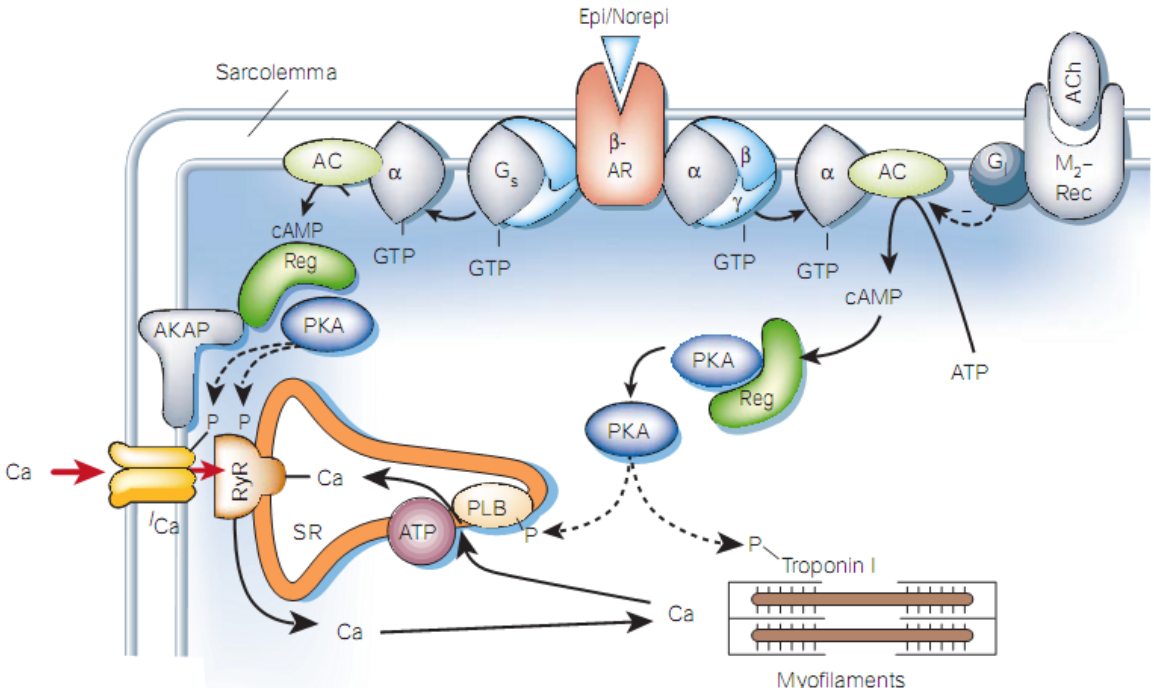


Figure 1-2 β-adrenergic receptor activation and phosphorylation targets relevant to excitation-contraction coupling. Inset shows the time course of an action potential, calcium transient and contraction measured in a rabbit ventricular myocytes at 37˚C. NCX, Na+/Ca2+ exchanger; ATP, ATPase; PLB, phospholamban; SR, sarcoplasmic reticulum.
This figure is obtained from Bers (2002) (Bers, 2002)

1.2.2.2. β-adrenergic signaling and cardiac arrhythmias

β1-stimulation leads to increased heart rate via stimulation of the I_f pacemaker current, which leads to spontaneous diastolic depolarization (Dorian, 2005). Heart rate is the key determinant of myocardial oxygen consumption and increased of heart rate (Habib, 1997), a phenomenon termed as tachycardia, substantially increases myocardial oxygen demand. Accordingly, clinical evidence suggests a strong association between increased heart rate and cardiovascular mortality in the acute (Reich et al., 2002) and chronic setting (Cook et al., 2006). Myocardial ischemia may occur if oxygen consumption outstrips demand, and lead to multiple secondary electrophysiologic changes that are known to be arrhythmogenic (Thomas et al., 2004). It was also shown that during ischemia, accumulation of catecholamines within the extracellular space of myocardium enhanced the stimulation of sympathetic nervous system (Schomig et al., 1984). This may in turn result in intracellular calcium overload and hence delayed afterdepolorizations (DAD), triggering the occurrence of cardiac arrhythmia (Eisner et al., 2009).

1.2.2.3. Calcium overload and arrhythmogenic calcium waves

The term 'Ca^{2+} overload' is applied to conditions in which Ca^{2+} waves and their consequences (DAD and aftercontractions) are observed (Venetucci et al., 2008). Much research has elucidated how Ca^{2+} overload and waves develop. The first stage is an increase in Ca^{2+} loading of the SR, which can arise because of increased loading of the cell with Ca^{2+} as a consequence of an imbalance between Ca^{2+} entry and efflux.(Trafford et al., 2001; Trafford et al., 1997) This will result in an increase of SR

Ca^{2+} content until a threshold level is reached at which waves are observed (Venetucci et al., 2007). It appears that the increased SR Ca^{2+} content results in an increased frequency of Ca^{2+} sparks and hence in the Ca^{2+} wave initiation (Cheng et al., 1996).

For a Ca^{2+} wave to occur, the Ca^{2+} released from a point in the SR must be able to diffuse through the cytoplasm and trigger another release from other region (MacQuaide et al., 2007). It has been suggested that the Ca^{2+} released by a single spark will be taken up by cytoplasmic buffers and therefore unable to activate further release. Once the wave has been initiated, the greater the SR Ca^{2+} content, the greater the amount released and the more likely a wave is propagated (Cheng et al., 1996). The SR Ca^{2+} threshold can be decreased or increased by enhanced or inhibited open probability respectively (Trafford et al., 2000). Diastolic Ca^{2+} waves are thought to underlie certain forms of arrhythmia as a result of some of the calcium in the wave being pumped out of the cell by NCX (Venetucci et al., 2008). The resultant NCX current may depolarize the cell and result in a DAD (Venetucci et al., 2008).

In summary, when SR Ca^{2+} content exceeds the critical SR threshold, Ca^{2+} wave is formed even in an unstimulated condition. This spontaneous Ca^{2+} release may then produce DADs and result in an action potential which causes ectopic beats and hence arrhythmias. As such, removal of Ca^{2+} overload is seen to be an important therapeutic strategy to treat Ca^{2+}-wave-dependent arrhythmias.

1.3. Ischemic Heart Disease

The 20th century saw significant increases in life expectancy and a major shift in the causes of illness and death throughout the world. During this transition, cardiovascular disease (CVD) became one of the most common causes of death worldwide. Before 1900, infectious diseases and malnutrition were the most common causes of death in the world. With improved nutrition and public health measures, both have declined significantly. Increased longevity and the impact of smoking, unhealthy diets, and other risk factors have combined to make CVD and cancer the leading causes of death in most countries, including Singapore. Today, it accounts for nearly 30% of deaths worldwide including about 40% in high-income countries and approximately 28% in middle- and low-income nations (Libby et al, 2008).

Cardiovascular disease covers wide array of disorders, such as disease of the cardiac muscle and of the vascular system supplying essential substances to heart, brain and other vital organs. The most common manifestations of CVD are coronary heart disease, congestive heart failure and stroke (Lopez et al, 2006).

1.3.1. Epidemiology

Ischemic heart disease, also called coronary heart disease, is one of the most common fatal diseases in the industrialized countries. In the United States, for instance, an estimated 17,600,000 American adults are living with ischemic heart disease (American Heart Association, 2010). This year, an estimated 785,000 people will suffer a new coronary attack and about 470,000 will have a recurrent coronary attack. The importance of coronary heart disease extends beyond the high morbidity and mortality

rates. Clinical manifestations are unpredictable or absent; and in 30~50% of patients, death is sudden and unexpected. The recognition of coronary heart decrease in any of its clinical forms raises the possibility of sudden death (Cheitlin et al., 1993).

1.3.2. Ischemia-reperfusion injury

Myocardial ischemia occurs when an atherosclerotic plaque that slowly builds up in the lumen of a coronary artery suddenly ruptures and blocks the blood flow downstream. Upon the obstruction, downstream myocardium is starved of oxygen and nutrients, where myocardial infarction (MI) develops (Reimer and Ideker, 1987). MI is a common presentation of ischemic heart disease. Most individuals with coronary heart disease show no evidence of narrowed artery for decades until the disease progresses to the advanced state when the first symptom, often a "sudden" heart attack, finally arise (American Heart Association, 2010).

The myocardium can tolerate short-term (up to 15 minutes) of myocardial ischemia without resulting cardiomyocytes death (Buja, 1998). During this short term ischemia episode, the defense mechanisms of heart seek to remedy this imbalance by decreasing myocardial contractile function and increasing the rate of glycolysis. (Braunwald and Kloner, 1982). Consequently, intracellular acidosis, resulted from the accumulation of glycolytic breakdown products, causes further inhibition of the contractile machinery (Heyndrickx et al., 1975). This phenomenon known as myocardial stunning, which is characterized by post-ischemic impairment of myocardial function, is considered acute and essentially reversible (Kloner and Jennings, 2001). With increasing duration of ischemia, greater irreversible myocardial damage could

develop upon a re-established blood flow to the blocked heart area, termed reperfusion injury (Yellon and Baxter, 2000).

Ischemic injury is a very complex process involving the action and interaction of many factors. Intensive investigation over decades has provided a detailed understanding of the complexity of the response of myocardium to an ischemic insult. Within ten seconds of blood flow interruption to the heart, mitochondrial oxidative phosphorylation rapidly stops, resulting in depletion of high-energy phosphate compounds, including ATP and creatine phosphate (Hearse, 1979). As a compensatory effect, anaerobic glycolysis increases to produce ATP but also leads to the accumulation of hydrogen ions and lactate (Buja, 2005). The resultant intracellular acidosis causes alterations in ion transport in the sarcolemma and organellar membranes (Buja et al., 1988; Thandroyen et al., 1992). Initially, there is increased K^+ efflux related to an increased osmotic load caused by the accumulation of metabolites and inorganic phosphate. With a significant decline in ATP, the Na^+, K^+-ATPase is inhibited, resulting in a further decrease of K^+ and an increase in Na^+. In addition, intracellular acidosis also activates the sarcolemmal Na^+–H^+ antiport (Karmazyn, 1999; Yellon and Baxter, 2000), which facilitates proton extrusion in exchange for Na^+. Collectively, this accumulated Na^+ in turn causes Na^+– Ca^{2+} exchanger to work in reverse mode, resulting in extrusion of Na^+ which brings in Ca^{2+} (White et al., 1984). The resultant cytosolic loading of Ca^{2+} not only induces sustained impairment on contractile function, but also mediates the damage on cell membrane, which leads to the progression of the injury to an advanced stage (Buja, 2005).

Ischemia also causes the depletion of glutathione (GSH), which is very important in maintaining cellular protein and lipid structure and functions by protecting these molecules from oxidation (Ji, 2002). Due to depletion of GSH, the toxic effects of oxidative stress are exacerbated (Patterson and Rhoades, 1988). The oxidative stress caused by ischemia result in an increased production and/or decreased degradation of reactive oxygen species (ROS), consisting of superoxide anion, hydrogen peroxide and hydroxyl radical, which are harmful metabolic by-products (Chang and Wu, 2006). ROS may initiate a chain reaction that results in irreversible changes in proteins or lipids. In the heart, ROS are also involved in many abnormalities, including cytotoxicity, cardiac stunning, arrhythmia, apoptosis, DNA break, and reduction of contractility (Takano et al., 2003). ROS impairs Na^+-K^+-ATPase activity, resulting in sodium overload, which further activate the Na^+-Ca^{2+} exchanger and lead to calcium overload in the sarcoplasmic reticulum (SR) ultimately. These changes collectively cause a loss of membrane integrity and terminally demolish the cell structure.

Although immediate restoration of blood flow and oxygen to ischemic tissue is ultimately beneficial, ischemic damage may be exaggerated upon reperfusion. This reperfusion injury is manifested by myocardial stunning, microvascular dysfunction and expedition of cell death in certain critically injured myocytes. In cellular level, reperfusion damage could be, in part, explained by calcium overload, oxygen free radicals and inflammatory processes (Maxwell and Lip, 1997; Park and Lucchesi, 1999).

In the ischemic myocardium, contracture develops through a rigor-type mechanism, leading to cytoskeletal defects. These defects result in a fragile and more

susceptible myocardium to mechanical damage during reperfusion (Schluter et al., 1996). During reoxygenation, ATP synthesis assists in cardiomyocyte recovery, but this process also re-activates the contractile machinery which leads to uncontrolled Ca^{2+}-dependent contraction (Schafer et al., 2001; Siegmund et al., 1997). This increased intracellular calcium at reperfusion may also lead to calcium overload, which in turn may cause delayed after-depolarization and ventricular automaticity (Opie and Coetzee, 1988). The overloaded calcium induces maximum contraction of the myofibrils upon reperfusion, resulting in a disruptive type of necrosis, termed contraction band necrosis (Verma et al., 2002). An increase in mitochondrial [Ca^{2+}] may also trigger the opening of mitochondrial permeability transition pore and lead to the release of cytochrome C and other pro-apoptotic factors that initiate the apoptotic cascade (Halestrap et al., 2004).

During reperfusion, oxygen is re-supplied to the myocardium, and undergoes a reduction process, resulting in superoxide anion formation (Di Paola and Cuzzocrea, 2007). Forming superoxide anion is the first step in the generation of other oxygen-derived reactive products, including hydrogen peroxide and hydroxyl radical (Park and Lucchesi, 1999). Neutrophils accumulate in the myocardium and become activated which in turn enhance oxygen free radical production (Chen et al., 1995). In the perfused myocardium, reoxygenated endothelial cells express adhesion proteins, release cytokines, and reduce production of NO which promotes adherence, activation, and accumulation of neutrophils in the ischemic-reperfused tissue (Ferrari et al., 1991; Jordan et al., 1999). These activated neutrophils will also release reactive oxygen species and proteolytic enzymes that can damage myocytes and vascular cells. In

addition, the newly returned blood also carries white blood cells including the neutrophils, releasing pro-inflammatory lipid metabolites which have been shown to enhance expression and production of a pro-inflammatory cytokine cascade involving interleukin 1 (IL-1) and tumor necrosis factor-α (TNFα) (Clark and Lutsep, 2001); these cytokines then lead to the generation of other pro-inflammatory molecules (such as IL-6, IL-8), activation and infiltration of leukocytes, and production of anti-inflammatory factors (including IL-4 and IL-10, which might produce a negative feedback on the cascade) (Jordan et al., 1999).

1.4. Clinical Treatment

1.4.1. First line

Myocardial infarction is a medical emergency which demands immediate attention and activation of the emergency medical services. Oxygen, aspirin (antiplatelet drug), glyceryl trinitrate (prodrug of NO) and morphine (analgesia), hence the popular MONA (morphine, oxygen, nitro, aspirin), are the first line drugs recommended to be administered as soon as the symptoms occur (Antman et al., 2004). Once diagnosed as myocardial infarction, the patient is also given other pharmacologic agents, including beta blockers, anticoagulation (typically with heparin), and possibly additional antiplatelet agents such as clopidogrel (Antman et al., 2004). Nevertheless, these agents are typically not given until the patient is evaluated by an emergency room physician or under the direction of a cardiologist.

1.4.2. Reperfusion therapy

The ultimate goal of the management in the acute myocardial infarction is to maintain the viability of as much myocardium as possible and prevent further infarction. Timely reperfusion of coronary flow facilitates cardiomyocyte salvage and improves their survival. Modalities for reperfusion include thrombolysis, percutaneous coronary intervention (PCI) and coronary artery bypass grafting (CABG).

Thrombolytic therapy achieves reperfusion by lysing the thrombi in the infarct artery. The effectiveness of thrombolytic therapy is determined by the timing of the therapeutic intervention. The best results are always observed when the thrombolytic agent is used within two hours of the onset of symptoms (Boersma, 2006). After 12 hours, associated risks like intracranial or systemic bleeding outweigh any benefit (LATE, 1993). An ideal thrombolytic drug would lead to rapid reperfusion, possess a high sustained patency rate, be specific for recent thrombi, be easily and rapidly administered, and create a low risk for intra-cerebral and systemic bleeding (White and Van de Werf, 1998). Currently available thrombolytic agents are streptokinase, urokinase, and alteplase (recombinant tissue plasminogen activator).

Percutaneous coronary intervention (PCI), commonly known as coronary angioplasty or simply angioplasty, is another effective procedure to treat the blocked coronary arteries by inflating a balloon within the artery to crush the thrombus. The procedure involves performing a coronary angiogram to determine the location of the blocked vessel, followed by balloon angioplasty to compress the plaque, and implantation of stents to prop the vessel open. The benefit of an immediate well-performed PCI over thrombolytic therapy has been well established (Grines et al., 1993;

Keeley et al., 2003). However, logistic and economic obstacles seem to hinder a more widespread application of PCI (Boersma, 2006).

Coronary artery bypass graft surgery is another important approach to salvage the blocked myocardium by reintroduction of blood supply. During the surgery, an artery or vein from elsewhere in the patient's body is grafted to the coronary artery to bypass narrowings or occlusions. Several arteries and veins can be used; however the left internal thoracic artery, usually grafted to the left anterior descending coronary artery (LAD), have been demonstrated to last longer than great saphenous vein grafts (Raja et al., 2004). Emergency CABG is less common than PCI for the treatment of an acute myocardial infarction. However, in patients with multiple coronary arteries occlusion, bypass surgery is a superior option when compared to PCI in terms of long-term survival rates (Hannan et al., 2005).

It is now known that irreversible injury occurs within 2–4 hours of the infarction, hence there is a limited time window for reperfusion to produce beneficial results. If attempts to restore the blood flow are initiated a few hours after a critical period, the result is deterioration instead of amelioration (Faxon, 2005). Moreover, reperfusion is unable to reverse the tissue damage. The lost cardiomyocytes will be replaced by a collagen scar that is not contractible and permanently impairs the contractile function of the heart. Accordingly, intense interest has been directed to investigate the application of stem cell for the repair of heart damage. However, the therapeutic application of this pioneering work on acute MI and post infarction treatment requires more research to prove its effectiveness and safety.

1.5. Experimental Therapy

1.5.1. Ischemic Preconditioning (IP)

Experimental and clinical studies have shown that a number of interventions, including brief periods of ischemia-reperfusion or hypoxia-reoxygenation and certain endogenous mediators or pharmacological agents are able to protect the heart against myocardial dysfunction, arrhythmias and infarction, three hallmarks of cardioprotection (Goto et al., 1995; Liem et al., 2002; Ryter et al., 2007).

In 1986 Murry and his colleagues published a landmark article in which they demonstrated that four repetitive 5-min episode of regional ischemia induced an extremely powerful protection against a subsequent lethal ischemia in anesthetized dogs. Infarct size was limited to 25% of that seen in the control group after 40 min of sustained ischemia (Murry et al., 1986). The investigators termed this phenomenon as 'ischemic preconditioning' (IP). Subsequently, numerous studies documented that IP is similarly effect in various other models (e.g., liver, kidney, brain, and endothelial cells), showing that short period(s) of ischemia or anoxia could allow tissues to survive subsequent ischemia that would have otherwise been lethal (Sanada and Kitakaze, 2004). Understanding this natural protection has since become one of the major targets in search for preventions against ischemic damages.

While initial studies demonstrated that IP could protect the heart against sustained ischemia that occurred soon after preconditioning, Kuzuya et al. and Marber et al. independently reported in 1993 that the cardioprotective effect of IP was still detectable 24 hours after preconditioning (Kuzuya et al., 1993; Marber et al., 1993). Kuzuya et al. also found that the cardioprotective effect of IP was lost between 3 and 12 hours after a

brief period of ischemia, indicating that there were two separate periods of cardioprotection afforded by IP. (Kuzuya et al., 1993) They named them the ‘‘first window’’ and ‘‘second window’’ respectively. The first window of the protection, often referred to as the classical or early phase, develops as early as few minutes after the preconditioning stimulus and lasts only 1–2 hours (Murry et al., 1986). The second window, also known as the late or delayed phase, develops 12–24 hours after the preconditioning stimulus, but lasts for 3–4 days (Kuzuya et al., 1993; Marber et al., 1993). The signaling mechanisms of these two phases are different. The first phase of protection is initiated by posttranslational modifications of proteins that are already present, whereas the second phase is mediated by synthesis of *de novo* proteins (Bolli, 2000). The early phase depends on actions that occur very rapidly, such as activation of ion channels or phosphorylation of enzymes, whereas the late phase involves longer time processes such as modulation of the genes regulating channel proteins, receptor, enzymes, molecular chaperon proteins, or immune factors (Sanada and Kitakaze, 2004). However, these two types of cardioprotection seem to share the similar triggers, mediators, and effectors despite the differences in the timing of participation in each cascade.

1.5.2. Ischemic Postconditioning

Despite the promising effect of IP, it has a major limitation which is that brief ischemia maneuver or its mimetic which can trigger pharmacological preconditioning has to be applied before the index ischemia insult. This has lead to the introduction of the concept: ischemic postconditioning (IPostC) by Na’s (Na et al., 1996) and Vinten-Johansen’s groups (Zhao et al., 2003). IPostC is defined as the phenomenon where rapid

intermittent interruptions of blood flow in the early phase of reperfusion resulted in a reduced myocardial injury (Zhao and Vinten-Johansen, 2006). This maneuver has produced promising protection against ischemia-reperfusion injury in mice (Kin et al., 2005), rats (Kin et al., 2004), rabbits (Yang et al., 2004b), dogs (Zhao et al., 2003) and in human patients (Staat et al., 2005).

The main purpose of introducing shuttering during initial reperfusion phase after ischemia is to disrupt a process known as lethal reperfusion injury. Lethal reperfusion injury occurs as a result of a sudden reflow of blood into the heart which leads to an abrupt change in the vascular environment. This results in detrimental events which subsequently lead to endothelial and vascular dysfunction, metabolic dysfunction, contractile dysfunction, dysrhythmias and eventually myocytes necrosis and apoptosis (Zhao and Vinten-Johansen, 2006). The mechanisms giving rise to the protective effect of IPostC constitutes two arms: passive and active (Tsang et al., 2005). The passive arm of IPostC refers to the mechanical events and cellular events directly resulted from the IPostC maneuver (Vinten-Johansen et al., 2005). The mechanical events interrupts the sudden onset of full flow reperfusion responsible for lethal reperfusion injury such as the negative modulation of coronary perfusion pressure whereas the cellular events involves the increased nitric oxide (NO) release due to better endothelial cell survival and preservation of their functions (Vinten-Johansen et al., 2005). In addition to the passive arm, IPostC treatment activates several pro-survival kinases such as Akt (Tsang et al., 2004; Zhu et al., 2006), Protein Kinase C (PKC) (Philipp et al., 2006; Zatta et al., 2006) extracellular signal regulated kinase 1/2 (ERK1/2) (Darling et al., 2005) and

inhibits Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) (Sun et al., 2006). These molecular events constitute the active arm of IPostC.

1.6. Hydrogen sulfide (H_2S)

Hydrogen sulfide (H_2S) has been known for hundreds years as a poisoning and toxic pollutant, and its best known effect is through the binding to cytochrome c oxidase which is responsible for its toxicity (Roth et al., 1995). However, the initial negative perception of H_2S has evolved with the recent discovery that it is produced by a number of enzymes in mammalian systems and modulates several physiological processes, and H_2S is now regarded as the third gasotransmitter alongside with nitric oxide (NO) and carbon monoxide (CO). In the following content, recent reports on H_2S will be reviewed with an emphasis on its biological functions and its roles in different diseases.

1.6.1. Physical and chemical properties of H_2S

H_2S is a colorless, flammable gas with a smell of rotten eggs. It is soluble in water (1 g in 242 ml at 20˚C) (Lim et al., 2008). H_2S is also soluble in organic solvents such as alcohol and ether, in addition to its good solubility in alkali carbonates and bicarbonates (Li et al., 2009). Its good solubility in lipophilic solvents (five fold greater than in water) allows H_2S freely penetrates cell membrane of all types. The detectable level of this gas by the human nose is at a concentration 400-fold lower (Wang, 2002) than the toxic level but prolonged exposure desensitize olfactory nerves and therefore renders the gas seemingly odorless to that individual (Li et al., 2009). Oxidation of H_2S yields

elemental sulfur, sulfur oxide (SO_2), and sulfates such as sulfuric acid (Wang, 2002). H_2S is weak acid which dissociates in water or plasma as follows:

$$H_2S \leftrightarrow HS^- + H^+ \leftrightarrow 2H^+ + S^{2-}$$

By using a standard Henderson-Hasselbach calculation, at physiological pH of 7.4, pKa of H_2S is 6.755 and 7.04, which render 18.5% and 30% of the total sulfide exists as the undissociated form in the buffer at 37˚C and 20˚C, respectively (Dombkowski et al., 2004).

1.6.2. Biosynthesis and catabolism of H_2S

1.6.2.1. Synthesis of H_2S

The biological production and utilization of H_2S have been well known for certain bacteria and archae (Pace, 1997). However, it is found that H_2S is formed in mammalian tissues by both endogenous enzymes and non-enzymatic pathways (i.e. via reduction of thiols and thiol-containing molecules) (Li et al., 2009). Most emphasis has been placed on the enzymatic formation of H_2S from L-cysteine by at least three pathways.

In the first pathway, two L-cysteine molecules undergo dimerization to form L-cystine, which is then transformed into thiocysteine, pyruvate and NH3 by cystathionine-γ-lyase (CSE, EC 4.4.1.1). CSE can further catalyze the reaction of thiocysteine with other thiols to form H_2S (Stipanuk and King, 1982; Yamanishi and Tuboi, 1981). In the second pathway, L-cysteine is hydrolyzed by cystathionine β-synthase (CBS, EC 4.2.1.22) to produce equimolar of H_2S and L-serine.(Li et al., 2009; Szabo, 2007) In the third pathway, cysteine aminotransferase (CAT, EC 2.6.1.3)

catalyzes the reaction of L-cysteine with a ketoacid to form 3-mercaptopyruvate may then be desulphurated by 3-mercaptopyruvate sulphurtransferase (3MST, EC 2.8.1.2) to form H_2S with a higher efficiency than CBS (Shibuya et al., 2009b). Of note, both CSE and CBS require pyridoxal 5'-phosphate (PLP) as cofactor to produce H_2S, whereas 3MST is zinc dependent (Li et al., 2009).

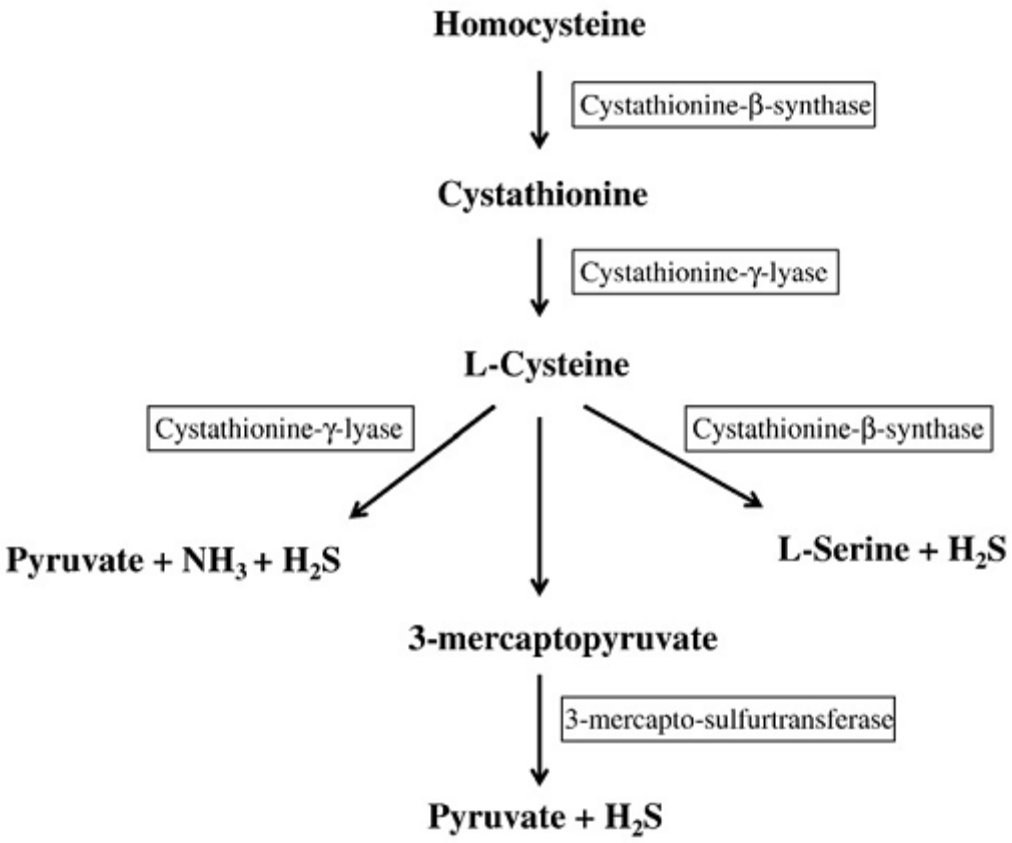


Figure 1-3 H2S can be synthesized by at least 3 metabolic pathways (Hughes et al., 2009)

1.6.2.2. Distribution of H_2S-genarating enzymes

The expression of CBS, CSE and 3MST has been detected in a board variety of cell types, including those from liver, kidney, thoracic aorta, ileum, portal vein, uterus, brain, pancreatic islets and the placenta (Nagahara et al., 1998; Shibuya et al., 2009a;

Shibuya et al., 2009b; Diwakar and Ravindranath, 2007; Hosoki et al., 1997; Kaneko et al., 2006; Kimura, 2010; Patel et al., 2009; Vitvitsky et al., 2006; Wang, 2002). In some tissues, both all three enzymes contribute to the local generation of H_2S, whereas in others, one enzyme predominates. CSE is primarily expressed in the heart, vascular and non-vascular muscles (Li et al., 2009; Lowicka and Beltowski, 2007) whereas CBS was thought to be the predominant H_2S-producing enzymes in brain and nervous system but the idea is recently challenged by the study showing that the H_2S level in the brain homogenates of CBS knockout mice showed no different from that of wild-type mice, suggesting that 3MST, which also expressed in brain, may be the main enzyme to generate H_2S in the system (Shibuya et al., 2009b).

1.6.2.3. Plasma and tissue H_2S level

The rate of H_2S production in tissue homogenates has been reported to be about 1 – 10 pmoles per second per mg protein, resulting in low micromolar extracellular concentrations (Doeller et al., 2005). Previous studies showed that both rat and human plasma contain approximately 50 μM of H_2S (Zhao et al., 2001), and an even higher level of H_2S (50~160 μM) was detected in the brain (Abe and Kimura, 1996). However, such remarkably high concentrations of H_2S have recently been questioned by some reviews (Lim et al., 2008; Szabo, 2007) and challenged by Whitfield *et al,* who found that the free H_2S gas was essentially undetectable (<100nM total sulfide) in all animals (Whitfield et al., 2008). In addition, they also found that H_2S is rapidly consumed when exogenous H_2S donor was added to blood (Whitfield et al., 2008), suggesting that H_2S is not transmitted via the circulation despite its significant local physiological and pathological roles in different systems. Indeed, over the past decade, most researchers

measured H_2S using a simple spectrophotometric assay which involves observation of color changes in acidifying zinc acetate-treated biological samples in the presence of a dye. Such a harsh condition may liberate sulfide from its bound forms, thereby producing concentrations that are likely to represent a mixture of free and bound sulfide. In addition, H_2S is either rapidly scavenged by hemoglobin or react with number of reactive oxygen species abundant in tissues, renders accurate measurements of such a reactive gas in biological tissues or circulation difficult.

1.6.2.4. Catabolism of H_2S

Comparatively to biosynthesis of H_2S, its catabolism is poorly recognized and most data were obtained by using exogenous H_2S (Lowicka and Beltowski, 2007) in which its physiological relevance is questionable. H_2S is rapidly oxidized, mainly in mitochondria, to thiosulfate, which is then converted to sulfite and sulfate. H_2S is excreted mainly by the kidney as free or conjugated sulfate (Beauchamp et al., 1984), however, it is notable that majority of sulfate in urine is believed to drive from the direct oxidation of cysteine by cysteine dioxygenase activity, as such, sulfate cannot be used as marker for the presence of H_2S. Methylation by thiol S-methyltransferase to methanethiol and dimethylsulfide (Beauchamp et al., 1984), and binding to methemoglobin to form sulfhemoglobin (Wang, 2002) may be the other two H_2S catabolism pathways in the cells.

1.6.3. Biological role of H_2S

1.6.3.1. H_2S and the central nervous system (CNS)

The presence of considerable amounts of H_2S and its synthase CBS in the brains of several species including humans suggested a role for this gas in CNS function (Abe

and Kimura, 1996). Increasing evidence shows that H_2S may have a role either as a neuromodulator or a neurotransmitter. H_2S has been shown to facilitate induction of hippocampal long-term potentiation by increasing the sensitivity of NMDA receptors (Kimura, 2000). Interaction of H_2S and NMDA receptors possibly involves a cAMP-dependent protein kinase pathway, since in the same study NaHS increases cAMP levels in neuronal and glial cell lines and primary neuron cultures. In addition, H_2S was also shown to elevate the intracellular calcium in microglial cells (Lee et al., 2006), astrocytes (Nagai et al., 2004) and both primary culture neuron (Garcia-Bereguiain et al., 2008) and neuronal cell line (Yong et al., 2010), further implying the neuromodulatory role of H_2S.

On the other hand, H_2S was reported to induce protection against glutamate-mediated toxicity in cortical neurons (Kimura et al., 2006) and mouse hippocampal cell line (Kimura and Kimura, 2004), perhaps by multiple mechanisms including activation of K_{ATP} and Cl^- channels and elevation of intracellular glutathione (Kimura et al., 2006). Lu *et al* also showed that H_2S may enhance glutamate uptake activity and thus protect primary cultured rat cortical astrocytes from H_2O_2-induced cellular injury via increase of ATP production and inhibition of ERK1/2 (Lu et al., 2008). Recently, NaHS was shown to be effective in treating and preventing neurotoxin-induced Parkinson's disease in rat via anti-oxidative stress and metabolic inhibition, highlighting the therapeutic potential of H_2S in neurodegenerative diseases (Hu et al., 2009).

Abnormal production of H_2S has been found to be associated with central nervous system diseases, such as stroke (Qu et al., 2006), Down syndrome (Kamoun et al., 2003), and perhaps also Alzheimer's disease (Beyer et al., 2004). In the rat model of

stroke (Qu et al., 2006), middle cerebral artery occlusion caused an increase in H_2S level in the lesioned cortex as well as an increase in the H_2S synthesizing activity. In keeping with this, administration of a sulfide donor significantly increased the infarct volume. In subjects with Down syndrome, urinary thiosulfate (a metabolite of H_2S) and erythrocyte sulfhemoglobin levels were both significantly increased compared with diet-matched controls (Kamoun et al., 2003). Further studies are warranted to determine whether the abnormality in H_2S level is a cause or simply a consequence of these diseases.

There is also growing interest in understanding the role of H_2S in pain appreciation. Administration of either NaHS (Distrutti et al., 2006a) or an H_2S-releasing derivative of mesalamine (Distrutti et al., 2006b) inhibited visceral nociception in the rat. Treatment with another H_2S donor, Lawesson's reagent, produces anti-nociceptive effect in joint inflammation (Ekundi-Valentim et al., 2010). Contradictorily, it was reported that intraplantar injection of H_2S in the hindpaw of the rat evokes pronociceptive activity (Kawabata et al., 2007), and intrathecal administration of NaHS causes hyperalgesia (Maeda et al., 2009) by an effect on T-type calcium channels, suggesting that H_2S may be a novel mediator for pain. More experiments are warranted to determine the factors behind the discrepancies in the studies, which may be closely related to the ambiguous role of H_2S in inflammation.

1.6.3.2. H_2S and Inflammation

Extensive studies have recently been conducted to define the role of H_2S in various inflammatory diseases. H_2S was shown to induce an upregulation of anti-inflammatory and cytoprotective genes including haem oxygenase-1 in pulmonary smooth muscle

cells *in vivo* (Qingyou et al., 2004) and in macrophages *in vitro* (Oh et al., 2006). H_2S also reduces LPS-stimulated TNF-α and NO formation in cultured microglial cells (Hu et al., 2007b). In animal models of inflammation, administration of H_2S donor has been effective in reducing carrageenan-induced paw edema and air pouch-induced leukocyte infiltration (Zanardo et al., 2006), the commonly-used systems to test the anti-inflammatory effects of experimental compounds. The protective effect of H_2S was attenuated by pretreatment with glibenclamide, suggesting the involvement of K_{ATP} channels. Several other studies demonstrated that chemically linking an H_2S-donor species to known anti-inflammatory drugs can improve the therapeutic profile of the compound. Using a rat model of endotoxin-induced inflammation, Li and colleagues reported that a sulfide-releasing compound, S-diclofenac, enhanced the anti-inflammatory effect of the parent molecule and exhibited less gastric toxicity (Li et al., 2007). Similarly, in the study by Distrutti et al., the H_2S-releasing derivative of mesalamine demonstrated superior anti-inflammatory efficacy compared with the base mesalamine molecule in the model of postinflammation hypersensitivity (Distrutti et al., 2006b).

However, it is intriguing that an enhanced H_2S-synthesizing activity or plasma H_2S level was observed in a large number of studies using different inflammation models. These include carrageenan-induced paw oedema in rats (Bhatia et al., 2005a), a mouse model of pancreatitis (Bhatia et al., 2005b), rodent model with endotoxic shock (Collin et al., 2005; Li et al., 2005), and a polymicrobial sepsis model in mice with cecal ligation and puncture (Marcotte and Walsh, 1975; Zhang et al., 2006). A pharmacological inhibitor of H_2S biosynthesis, DL-propargylglycine (PAG) (Marcotte

and Walsh, 1975), was used in some of these studies and shown to be able to attenuate the inflammatory responses. In a rat model of endotoxemia, PAG prevented the increases in the serum levels of liver and pancreas injury markers and reduced the tissue content of myeloperoxidase (Collin et al., 2005). In a model of cecal ligation and puncture, PAG treatment reduced tissue neutrophil infiltration and improved liver and lung histology (Marcotte and Walsh, 1975; Zhang et al., 2006). In a carrageenan-induced inflammation model in the rat, PAG treatment dose-dependently reduced paw edema and neutrophils infiltration (Bhatia et al., 2005a).

Interestingly, both inhibitors and donors of H_2S were shown to exert beneficial effects in the same experimental model of disease, for instance, in the carrageenan paw edema model (Bhatia et al., 2005a; Sidhapuriwala et al., 2007; Zanardo et al., 2006). While two studies demonstrate anti-inflammatory effects of H_2S (Zanardo et al., 2006), another one argues for a pro-inflammatory role of H_2S in the same model (Bhatia et al., 2005a). It is now generally accepted that these conflicting results are due to the opposite effect exerted by H_2S at low and high local concentrations, as well as the different speed of H_2S release from NaHS and H_2S-releasing moieties. (Li et al., 2007; Wallace, 2007) A similar paradox has been previously noted with inhibitors versus donors of NO — both of them being effective in the carrageenan paw edema models (Fernandes et al., 2002; Handy and Moore, 1998). Clearly, there is an exquisite balance and complex regulation and interaction of pathophysiological responses by endogenous and exogenous gasotransmitters (Szabo, 2007).

1.6.3.3. H_2S and cardiovascular system

Earlier, it was perceived that H_2S interfered with cardiovascular function as a result of the secondary anoxia rather than a direct action of the gas on cardiac myocytes or vascular smooth muscle cells (SMCs) (Reiffenstein et al., 1992). However, this view has been overturned by the finding of a detectable amount of H_2S and its synthase CSE in the cardiovascular system.

As early as in 1997, expression of CSE and endogenous production of H_2S have been detected in rat portal vein and thoracic aorta (Hosoki et al., 1997) followed by the study which revealed that CSE is the only H_2S-generating enzyme in rat mesenteric artery and other vascular tissues, with expression levels of CSE mRNA ranked in an order of pulmonary artery > aorta > tail artery > mesenteric artery (Zhao et al., 2001). On the other hand, Chen et al. found no activity or expression of CBS in human atrium and ventricle tissues (Chen et al., 1999). The activity and/or expression of CBS were also lacking in human internal mammary arteries, saphenous veins, coronary arteries, or aortic arteries (Bao et al., 1998; Chen et al., 1999). Thus, CSE appears to be responsible for the generation of H_2S in cardiovascular tissues specifically.

The effect of H_2S on vascular systems has recently been investigated in several in-depth studies. H_2S at physiologically relevant concentrations induces relaxation in portal vein (Hosoki et al., 1997), aorta (Zhao et al., 2001), and mesenteric artery beds of rats (Cheng et al., 2004). A previous study by Zhao et al. revealed that an intravenous bolus injection of H_2S at 2.8 and 14 μmol/kg body weight provoked a transient decrease in mean arterial blood pressure of anaesthetized rats (Zhao et al., 2001). At the tissue level, H_2S induced a concentration-dependent relaxation of the phenylephrine (PE)-

precontracted rat aortic tissues (IC_{50}, 125 μM). They also found that when isolated rat aortic tissues were precontracted with 20 or 100 mM KCl, the maximum vascular relaxation induced by H_2S was 90% and 19%, respectively. This difference in relaxation potency of H_2S may represent the portion of relaxation possibly mediated by potassium conductance. Furthermore, the effect of H_2S on aortic tone was only antagonized by the K_{ATP} channel blocker, glibenclamide, but not blockers of other types of potassium channels, indicating that the vasorelaxant effect of H_2S was K_{ATP}-dependent. Meanwhile, they demonstrated that H_2S directly increased K_{ATP} channel currents and hyperpolarized membrane in isolated SMCs.

In spite of the detection of CSE in myocardial tissues, the effect of H_2S on heart was relatively unclear. Previous studies showed that H_2S was involved in the delayed cardioprotection induced by ischemic preconditioning, possibly via activating sarcolemmal K_{ATP} channels, provoking NO release (Pan et al., 2006), enhancing expression of different PKC isoforms (Pan et al., 2007) and COX-2 (Hu et al., 2008a). However, the effect of H_2S in the immediate protection induced by ischemic preconditioning and postconditioning remains unclear.

Geng et al. observed a negative inotropic effect of H_2S in both *in vitro* and *in vivo* experiments, and the effect could partly be blocked by glibenclamide (Geng et al., 2004b). As discussed in the report, the cardiac contraction could also be affected by its peripheral vascular effect, because H_2S dilates blood vessels, which leads to reduction of central venous pressure, resulting in a decrease of the venous return and reduction of cardiac pre- and post-loads. Although this study is suggestive of a role for H_2S in regulating cardiac function, more experiments are required to determine the role of H_2S

in intracellular calcium and cellular contraction, in order to examine the detailed mechanisms.

Accordingly, the aims of the current study are to investigate the potential role of hydrogen sulfide in regulating the heart functions via interaction with beta-adrenergic system and nitric oxide, another very important gaseous transmitter, under the physiological and pathological conditions. By using different methods and disease models, the involvement of hydrogen sulfide in cardioprotection conferred by ischemic pre- and post-conditioning were also examined.

As used in numerous publications, NaHS, a donor of H_2S, was employed in the experiments in the following chapters as its use allows for a better determination of the concentration of H_2S in solution than bubbling H_2S gas. NaHS dissociates to Na+ and HS- in solution. Thereafter HS- associates with H+ and produces H_2S. Approximately one-third of the H_2S in aqueous solution exists in the undissociated form (H_2S) at 20 ºC (Abe and Kimura, 1996). At 37 ºC, the undissociated form of H_2S is around 18.5% (Dombkowski et al., 2004) i.e. approximately 18.5 μM when 100 μM NaHS was applied, which is well within the range of physiological concentrations in rat plasma (Zhao et al., 2001). It is notable that 100 μM NaHS does not alter the pH of the buffers (Dombkowski et al., 2005). For this reason, NaHS has been widely used for studies on H_2S (Distrutti et al., 2006a; Dombkowski et al., 2005).

Chapter 2 Negative regulation of β-adrenergic function by hydrogen sulfide in the rat heart

2.1. Introduction

Hydrogen sulfide (H_2S) represents the most recently identified endogenous gaseous messenger (Wang, 2002). The detection of plentiful H_2S synthase CSE in the heart (Geng et al., 2004b) suggests that endogenous H_2S production is important for a well-functioned heart. However, it is not clear what the role of H_2S is in pathological conditions. Thus I investigated whether there is a difference in endogenous H_2S production between healthy cardiomyocytes and myocytes undergoing ischemia challenge.

The β-adrenergic signaling pathway is the primary mechanism that is able to transiently increase cardiac output. β-adrenergic receptors are transducers linking hormone-mediated chemical signals to the mechanical event of augmented myocardial contraction (Dorian, 2005). In cellular level, the classic route of contractile function stimulation is the result of the activation of adenylyl cyclase (AC) catalytic activity by the β-adrenoceptor-coupled stimulatory G protein, which leads to increased intracellular cAMP level. This in turn stimulates protein kinase A (PKA), which then mediates phosphorylation of L-type Ca^{2+} channels leading to Ca^{2+} influx to the intracellular compartment. Ca^{2+} entry triggers Ca^{2+} release from sarcoplasmic reticulum and the elevated free intracellular Ca^{2+} ($[Ca^{2+}]_i$) bind to the myofilament protein troponin C, which then switch on the contractile machinery (Bers, 2002).

Of note, it was reported that H_2S production is markedly decreased during over-stimulation of β-adrenergic system (Geng et al., 2004a). In addition, H_2S was shown to

decrease heart contractile function including heart contractility, left-ventricular pressure development and left-ventricular-end systolic pressure (Geng et al., 2004b). Bearing in mind that the contractile state of the heart is under direct control of the adrenergic nervous system, I hypothesized that H_2S may modulate the β-adrenergic system and this modulation of the β-adrenergic receptor (β-AR) may be of pathological importance during ischemia, which causes cardiac injury and arrhythmia due to calcium overload.

2.2. Materials and methods

All experimental protocols mentioned in this and the rest of the chapters were approved by the Institutional Animal Care and Use Committee of the National University of Singapore

2.2.1. Isolation of adult rat cardiomyocytes

Sprague-Dawely rats (220-300 g, male) were anesthetized with intraperitoneal (*i.p.*) injection of a combination of ketamine (75mg/kg) and xylazine (10mg/kg). Heparin (1000 IU) was administered *i.p.* to prevent coagulation during removal of the heart. The heart was quickly excised, mounted on a Langendorff apparatus, and perfused in a retrograde fashion via the aorta with calcium-free Tyrode's solution (in mmol/L): 137 NaCl, 5.4 KCl, 1 $MgCl_2$, 10 HEPES, 10 Glucose, pH 7.4 at 37 °C. After 5 min the perfusion solution was changed to the Tyrode's solution containing 1 mg/ml collagenase type I and 0.28 mg/ml protease (type XIV) and perfused for a further 25-30 min. The perfusion solution was then changed to Ca^{2+}-Tyrodes solution containing 2×10^{-4} mol/L $CaCl_2$ without enzymes for an additional 5 min. The ventricular tissue was then

cut into small pieces in a Petri dish containing pre-warmed Ca^{2+} Tyrode's solution and shaken gently to ensure adequate dispersion of dissociated cardiac myocytes. A 2.5×10^{-4} meter mesh screen was used to separate the isolated cardiac myocytes from cardiac tissue. The cells were then washed three times in Ca^{2+}-Tyrode's solution and collected by centrifugation (500 rpm, for 1 min). Ca^{2+} concentration of the Tyrode's solution was increased gradually to 1.25×10^{-3} mol/L in 20 min. More than 70% of the cells were rod-shaped and impermeable to trypan-blue. The cells were allowed to stabilize for 30 min before any experiments.

2.2.2. Measurement of H_2S concentration

The culture media of cardiomyocytes were collected for measurement of endogenous H_2S production. 75 μL media from each sample was added into an Ependorff tube that already contained 450 μL deionised water and 250 μL zinc acetate (1% w/v). Then *N*, *N*-dimethyl-*p*-phenylenediamine sulphate (20 μM in 7.2 mol/L HCl, 133 μl) and $FeCl_3$ (30 μM in 1.2 mol/L HCl, 133 μL) were added in sequence for color development at room temperature. After 10 min, trichloroacetic acid (10% w/v, 250 μL) was added to precipitate any protein that might be present in the media. The tubes were then centrifuged (10,000 × g) for 3 min and 300 μl aliquots from the resulting supernatants were transferred into a 96-well plate. Absorbance was determined at 670 nm using a 96 well microplate reader (Tecan Systems Inc., U.S.A.).

2.2.3. Measurement of contractile and relaxation function

Twitch amplitudes of cells were recorded and measured as described previously.(Hu et al., 2007a) Briefly, cell images were also continuously monitored through a x40 objective lens (Nikon, Singapore) and transmitted to a charge-coupled device (CCD)

black and white (B/W) video camera (NL-2332; National Electronic, Canada). The output from the CCD camera was displayed on a video monitor (National Electronic, Canada). Myocyte edge was measured using a video motion edge detector (VED-105; Crescent Electronics, Canada). Light-dark contrast of the edge of the myocytes provides a marker for measurement of the amplitude of motion. The amplitude of marker was directly proportional to the dark image of contraction and the action was in real time. The amplitude of myocyte motion remained unchanged for at least 10 min, indicating the stability of the preparation. The data collection time point was set to be 1000 sec after drug treatments, since maximum response reached at less than 900 sec after drug administration. This was also applied to calcium transient amplitude data collection throughout the study. The maximum velocity of cell shortening (-dL/dt) and relaxing (+dL/dt) is represented by average value of maximum changes of length/time of at least five continuous tracing in each cell, at 1000 s after each treatment.

2.2.4. Measurement of intracellular Ca^{2+} ($[Ca^{2+}]_i$)

Ventricular myocytes were incubated with fura-2/AM (4×10^{-6} mol/L) (25 min) in Tyrode's solution supplemented with 1.25×10^{-3} mol/L $CaCl_2$. The unincorporated dye was removed by washing the cells twice with fresh incubation solution. Loaded cells were kept at room temperature (24 °C-26 °C) for 30 min to allow the fura-2/AM in the cytosol to de-esterify.

Loaded ventricular myocytes were then transferred to the stage of an inverted microscope in a superfusion chamber at room temperature. The inverted microscope was coupled with a dual-wavelength excitation spectrofluorometer (Intracellular imaging Inc, USA). Myocytes were perfused with Krebs' bicarbonate buffer (KB buffer,

mmol/L; 117 NaCl, 5 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 1.25 $CaCl_2$, 25 $NaHCO_3$, 11 glucose, with 1% w/v dialyzed BSA) and gassed with 95% O_2/5% CO_2. The myocytes selected for the study were rod-shaped with clear striations. These cells exhibited synchronous contraction (twitch) in response to suprathreshold (4 ms, 0.2 Hz) stimuli delivered by a stimulator (Grass S88, Canada) via two platinum field-stimulation electrodes immersed in the bathing fluid. Fluorescent signals obtained at 340 nm (F340) and at 380 nm (F380) excitation wavelengths were stored in a computer for data processing and analysis. The F340/F380 ratio was used to indicate $[Ca^{2+}]_i$ level in the myocytes.

2.2.5. Assay of cAMP

The cAMP produced in the cells from different treatment groups were sampled and measured according to the instruction provided in the direct cAMP enzyme immunoassay kit purchased from Sigma-Aldrich (St. Louis, MO, USA). The pellets were stored at -20ºC for protein determination. Data were compared to a standard curve to obtain the actual cAMP concentrations.

2.2.6. Cell fractionation and adenylyl cyclase activity assay

A cell fractionation technique was adopted from the literature (Mackay and Mochly-Rosen, 2001a; Weber et al., 2005) and described in our previous publication (Pan et al., 2007). Adenylyl cyclase activity was assayed as described previously with some modifications (Lin et al., 2002). The adenylyl cyclase (AC) activity assay was performed at 37ºC for 10 min in a 400 μL reaction mixture containing 1 mM ATP, 100 mM NaCl, 50 mM Hepes, 0.5 mM 3-isobutyle-1-methylxanthine, 6 mM $MgCl_2$, 1 μM GTP, and 20 μg of membrane protein. In forskolin group, samples were treated with

forskolin (100 μM) for 10 min. In Forskolin+NaHS group, NaHS (100 μM) was given 5 min before and during forskolin treatment. Reactions were stopped by addition of 0.6 mL of trichloroacetic acid (10% w/v). The accumulation of cAMP was later assayed by cAMP EIA kit (Cayman Chemical, USA).

2.2.7. Statistical analysis

Values presented are mean ± standard error of mean. SPSS for Windows (11.5.0, LEAD technologies, USA) was used to perform the analysis. One-way analysis of variance (ANOVA) was used to determine the difference among groups. A post hoc Bonferroni analysis was used to determine the significant differences between the groups. The significance level was set at $P<0.05$.

2.2.8. Drugs and Chemicals

Type 1 collagenase, protease XIV, isoproterenol, forskolin, 8-bromoadenosine-3',5'-cyclic monophosphate, NaHS, and N, N-dimethyl-p-phenylenediamine sulphate, FeCl3 isobutyle-1-methylxanthine and caffeine were purchased from Sigma Chemical Co, USA. Fura-2 was purchased from Molecular Probes Inc. USA. Bay K-8644 and GTP were purchased from Calbiochem (USA).

2.3. Results

2.3.1. Effect of NaHS on isoproterenol-augmented contraction in electrically-stimulated ventricular myocytes.

Figure 2-1A shows the representative tracings of the effect of ISO (0.5 μM) on myocyte contraction in the presence and absence of NaHS (100 μM). Stimulation of β-adrenoceptor with ISO significantly increased the twitch amplitude (Figure 2-1B), maximal velocity of cell shortening (+dl/dt), and maximal velocity of cell relaxing (-dl/dt, Figure 2-1C) of the single ventricular myocytes. Pretreatment with NaHS (100 μM) for 5 min, which itself had no effect, significantly attenuated the effect of ISO (0.5μM) on these parameters (Figure 2-1B & 2-1C) ($n = 7$. $p<0.001$ for ISO vs control group; $p<0.01$ or 0.05 for NaHS+ISO vs ISO group). The concentration-dependent effect of ISO on myocyte twitch amplitude was shown in Figure 2-1D. ISO at 10^{-10} to 10^{-6} M increased the electrically-induced contraction of the single ventricular myocytes in a concentration-dependent manner. The maximal effect of ISO (Emax) was 86.4 ± 8.6% of control (before ISO treatment) and the EC50 value was 60.1 nM. Pretreatment with NaHS (100 μM) for 5 min significantly attenuated the effect of ISO at a concentration range from 5×10^{-8} to 10^{-6} M. ($n = 6$-7, $p<0.05$ or 0.01, Figure 2-1D). The Emax and EC50 of ISO (Emax) were decreased to 50.7 ± 8.9% and 5.68 nM, respectively, in NaHS treatment group. The concentration-dependent effect of NaHS was shown in Figure 2-1E, NaHS at 10^{-5} to 10^{-3} M concentration-dependently attenuated ISO (0.5 μM)-augmented cell contraction ($n = 6$-7, $p<0.05$ or 0.01, Figure 2-1E). Taken together, these data suggest that H_2S attenuates the myocytes twitch amplitude enhanced by β-adrenoceptor-stimulation.

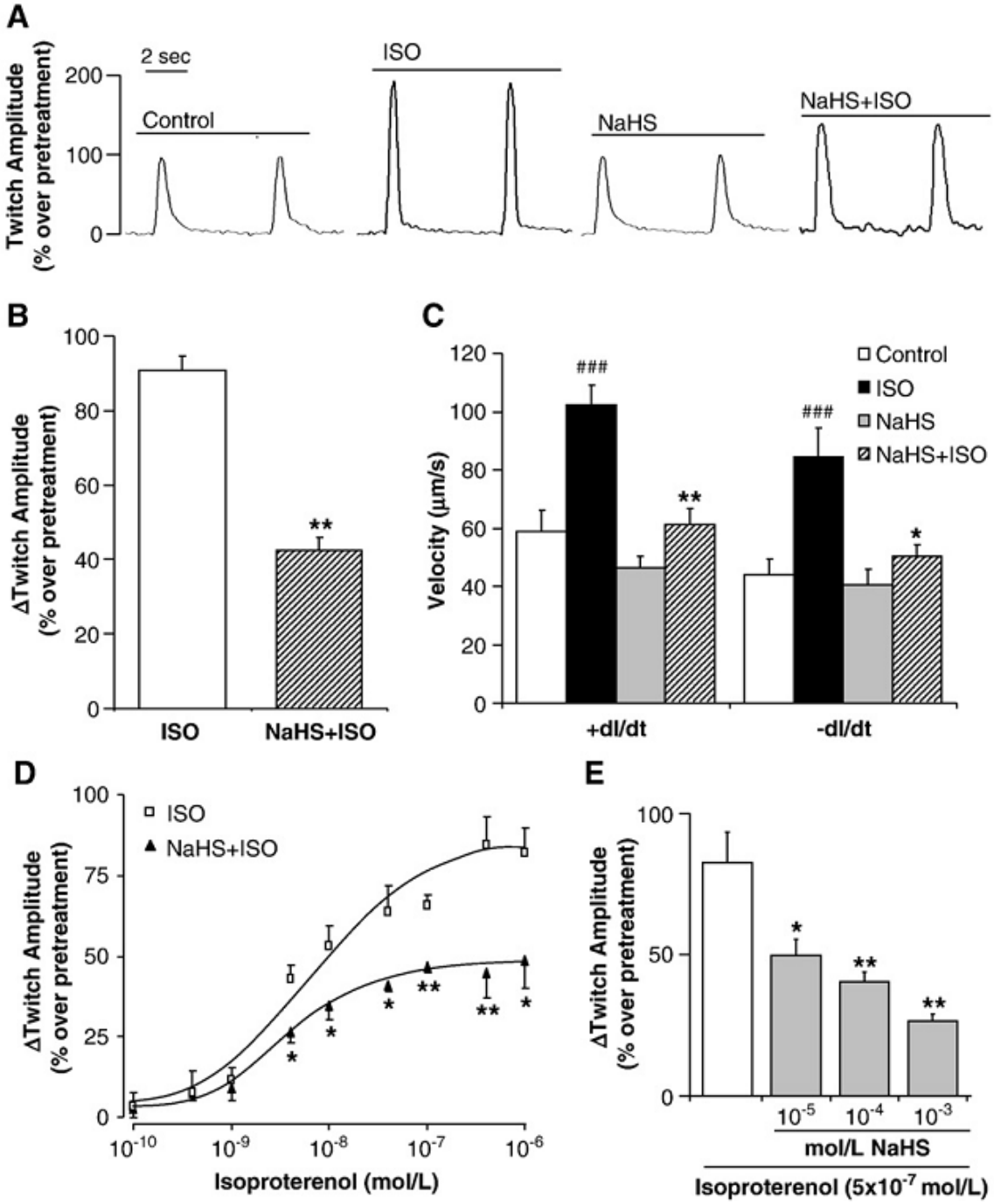


Figure 2-1 Inhibitory effect of NaHS on ISO augmented contraction in electrically-stimulated rat ventricular myocytes. (A) Representative tracings of myocytes twitch amplitude of control, ISO (0.5 μM), NaHS (100 μM) and NaHS+ISO treatment group. (B & C) Group data showing that NaHS (100 μM) attenuated the effect of ISO (0.5 μM) on twitch amplitude (B) and maximal velocity of cell shortening and relaxing (C). Mean±S.E.M, n = 7 for each group, $^{\#\#}p<0.01$, $^{\#\#\#}p < 0.001$ vs control; *p<0.05, **p< 0.01 vs ISO group. (D) Concentration-dependent effect of ISO on myocyte twitch amplitude in the presence and absence of NaHS. The cells were treated with NaHS (100 μM) for 5 min before administration of ISO at the concentration range from 10^{-10} to 10^{-6} M. Mean±S.E.M, *p<0.05 and **p<0.01 vs corresponding value in ISO group. (E) Concentration-dependent effect of NaHS (10^{-5} to 10^{-3} M) on ISO (0.5 μM)-augmented contraction in the electrically-stimulated ventricular myocytes pretreated with NaHS. Mean±S.E.M, n = 6-7, *p<0.05 and **p<0.01 vs ISO alone group.

2.3.2. Effect of NaHS on ISO-augmented $[Ca^{2+}]_i$ transients in electrically-stimulated ventricular myocytes

To determine the effect of H_2S on calcium handling, I observed the effect of H_2S on the amplitude of electrically-induced (EI) $[Ca^{2+}]_i$ transients upon ISO treatment. As shown in Figure 2-2A, the amplitude of electrically-induced $[Ca^{2+}]_i$ transients was significantly augmented by ISO (0.5 μM), which was attenuated by pretreatment with NaHS (100 μM) for 5 min (Figure 2-2B). Data are presented as $\Delta[\ Ca^{2+}]_i$ transient amplitude by measuring the difference of $[Ca^{2+}]_i$ transient amplitudes between before and after drug treatment. Figure 2-2C shows that NaHS significantly attenuated the effect of ISO on electrically-induced $[Ca^{2+}]_i$ transients ($\Delta[Ca^{2+}]_i$ transient amplitude: ISO group: 0.46 ± 0.07; NaHS+ISO group: 0.06 ± 0.01; n = 5, $p<0.05$, Figure 2-2C).

In order to assess the involvement of sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA) in this inhibitory regulatory effect of H_2S, I assessed the decline rate of EI-$[\ Ca^{2+}]_i$, an indicators of SR- Ca^{2+} uptake rate.(Pan et al., 2007) Figure 2-2D and 2-2E shows that both t50 (half-decay time) and t90 (90%-decay time) were greatly shortened by ISO treatment. Despite attenuating the effect of ISO on cell shortening and relaxing velocities, H_2S failed to alter the SR- Ca^{2+} uptake rate accelerated by ISO. These data suggest that the effect of H_2S on myocyte contraction is not via affecting the Ca^{2+} uptake by SERCA.

I also observed whether NaHS changes the calcium response kinetics to ISO. The maximum calcium response of myocyte to ISO reached within 900 sec after treatment. The EI-$[\ Ca^{2+}]_i$ transient peak/time curve (shown by arrows in Figure 2-2A-B) upon ISO treatment was well fitted with the Boltzmann sigmoidal function. I found that NaHS did not significantly change the time to reach 20% (t20), 50% (t50) and 80%

(t80) of maximum response, suggesting that NaHS had no effect on the response kinetic profile of ISO treatment (Figure 2-2F). Taken together, our data indicate that the inhibitory effect of H_2S appears to be primarily via modulation of the β-adrenergic system rather than via regulation of myofilament function.

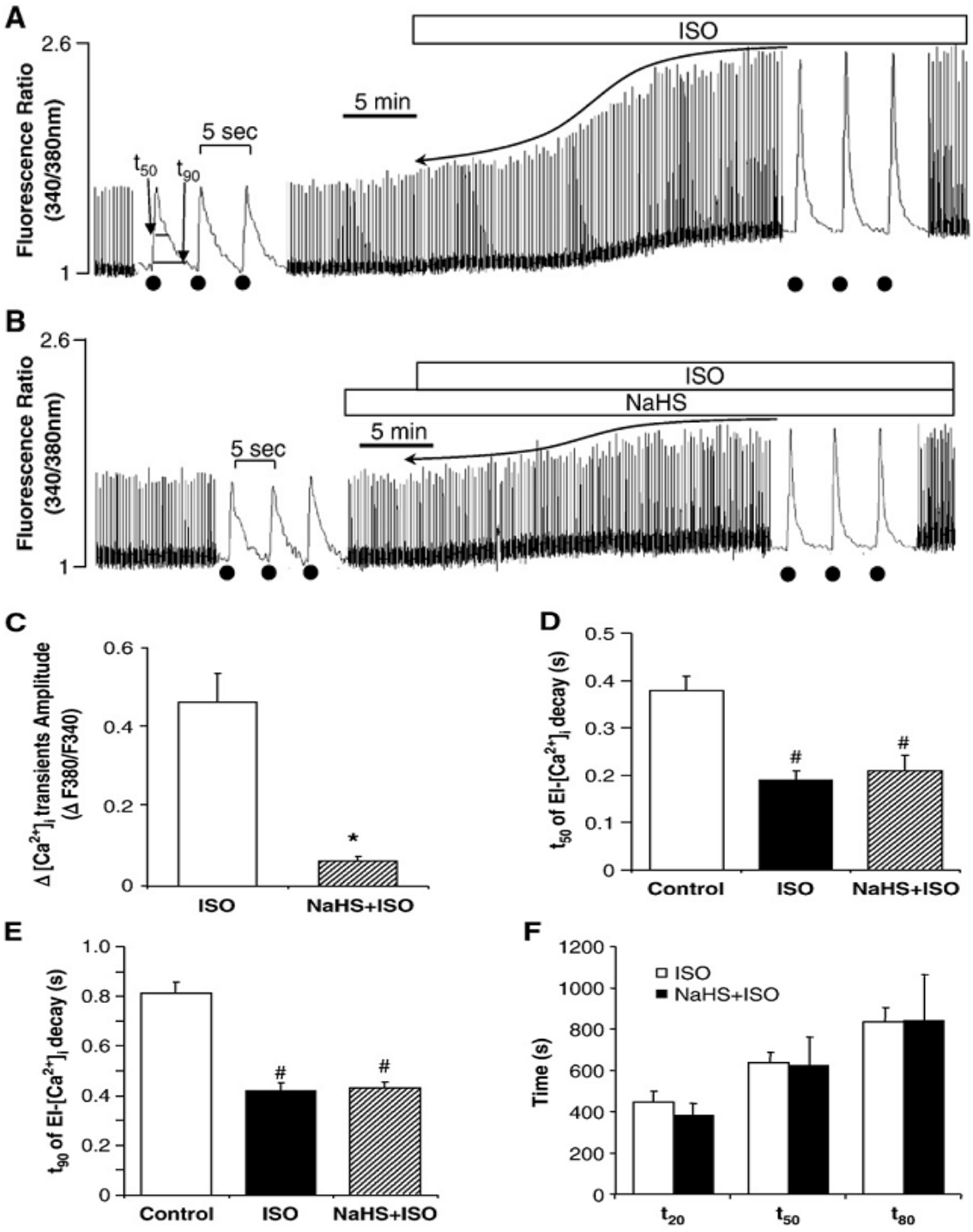


Figure 2-2 Inhibitory effect of NaHS on ISO-augmented $[Ca^{2+}]_i$ transients in the electrically-stimulated ventricular myocytes. (A-B) Representative tracings showing the effect of 0.5 μM ISO on $[Ca^{2+}]_i$ in the ventricular myocytes pretreated with (B) and without (A) of 100 μM NaHS. The time scale for time-expanded section and compressed section are indicated separately. • Electrical stimulation, each • is separated by 5 sec. (C - F) Group data showing the effect of NaHS (100 μM) on ISO-augmented calcium transient amplitudes (C), decay time of EI-$[Ca^{2+}]_i$ transients (D, t50: half-decay time; E, t90: 90%-decay time) and calcium response kinetics to ISO (F, t_{20}, t_{50} and t_{80} indicate the time to reach 20%, 50% and 80% of maximum response respectively) and Mean±S.E.M, n = 5, $^{\#}p<0.05$ vs control, $^{*}p<0.05$ vs ISO group.

2.3.3. Effect of NaHS on forskolin-augmented $[Ca^{2+}]_i$ transients and contraction in electrically-stimulated ventricular myocytes

Stimulation of β-adrenergic system activates adenylyl cyclase. To determine whether H_2S acts on adenylyl cyclase, forskolin (an activator of adenylyl cyclase) was used. Forskolin (0.5 μM) significantly increased the amplitudes of electrically-induced $[Ca^{2+}]_i$ transients (Figure 2-3A) and myocyte twitch (Figure 2-3C) of the single ventricular myocytes. Pretreatment with NaHS (100 μM) attenuated these effects ($\Delta[Ca^{2+}]_i$ transients amplitude: Forsk group: 0.26 ± 0.05%; NaHS+Forsk group: 0.05 ± 0.02%; n = 5, $p<0.05$; Δtwitch amplitude: Forsk group: 60.4 ±1%; NaHS+Forsk group: 39.3 ± 5%; n = 5, $p<0.01$, Figure 2-3E). Figure 2-3F and 2-3G show that H_2S attenuated the effect of forskolin on ±dl/dt but not on EI-$[Ca^{2+}]_i$ decay. There was no significant difference in t20, t50 and t80 between forskolin and forskolin+NaHS groups (Figure 2-3H), suggesting that the response kinetics of forskolin did not differ between the groups with or without treatment of NaHS. These data indicate that H_2S may act on adenylyl cyclase or its downstream effectors.

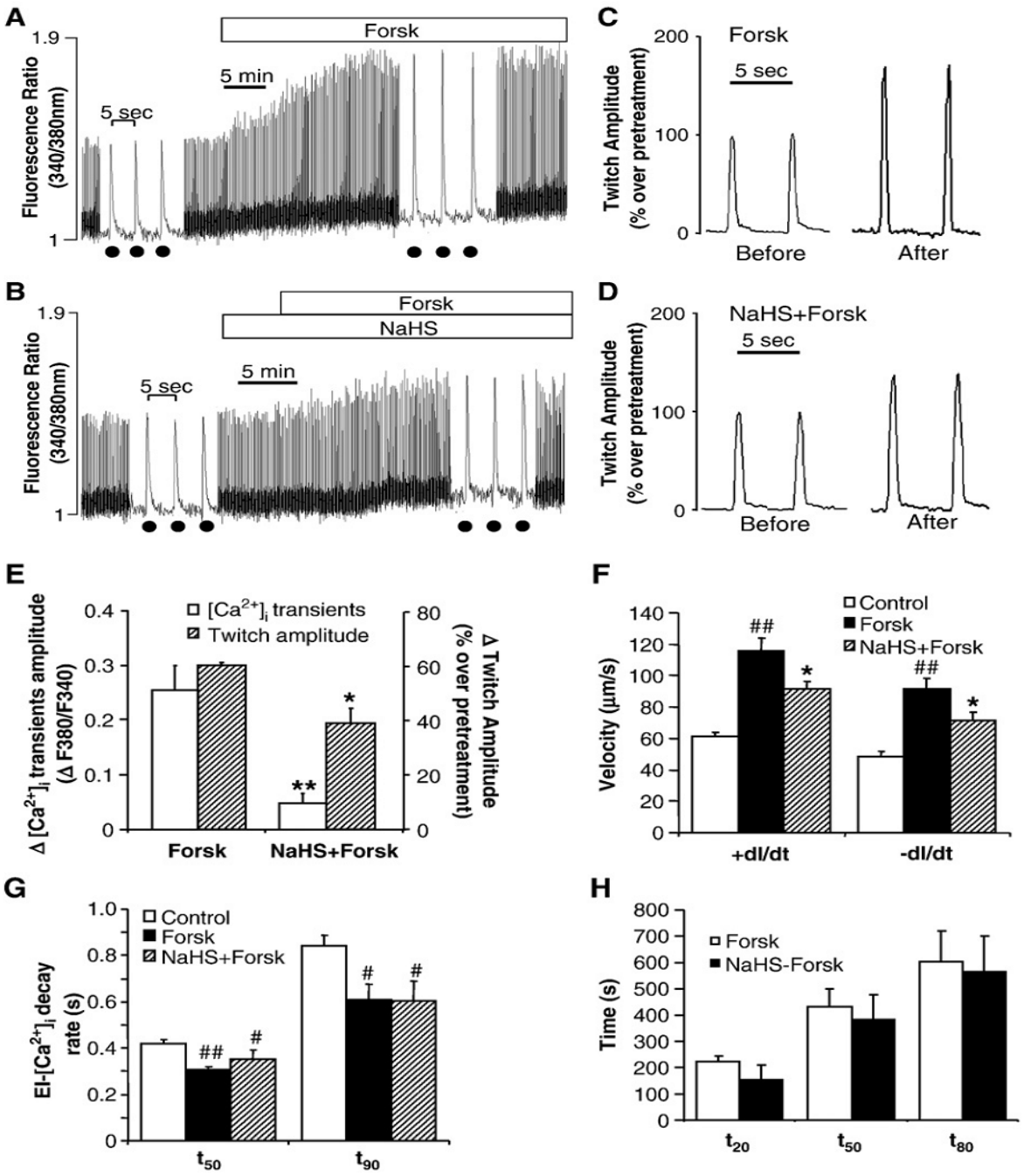
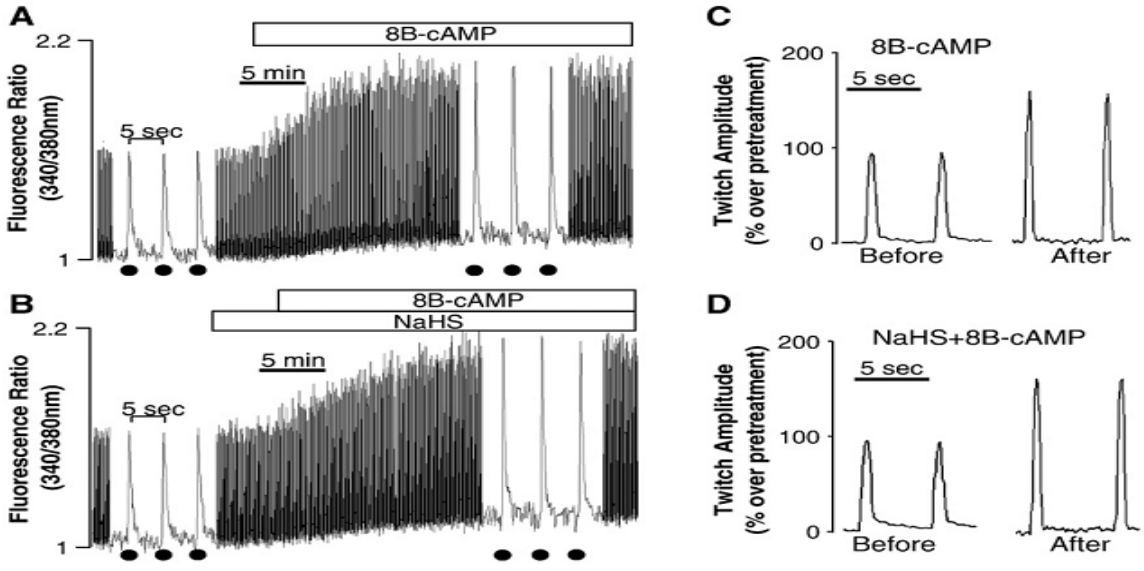


Figure 2-3 Inhibitory effect of NaHS on forskolin augmented $[Ca^{2+}]_i$ transients and twitch amplitude in the electrically-stimulated ventricular myocytes. (A-D) Representative tracings showing the effect of 0.5 μM forskolin on $[Ca^{2+}]_i$ transients (A & B) and myocyte twitch amplitude (C & D) in the ventricular myocytes in the absence (A & C) and presence (B & D) of 100 μM NaHS. The time scale for time-expanded section and compressed section in $[Ca^{2+}]_i$ transient tracings are indicated separately. • Electrical stimulation, each • is separated by 5 sec. (E-H) Group data showing the effect of NaHS (100 μM) on forskolin-augmented amplitudes of electrically-induced $[Ca^{2+}]_i$ transients and myocyte twitch (E), maximal velocities of cell shortening and relaxing (F), decay rate of EI-$[Ca^{2+}]_i$ transients (G) and calcium response kinetics (H). Mean±S.E.M, n = 5, *p<0.05, **p<0.01 vs corresponding values in forskolin group; $^{\#}$p<0.05, $^{\#\#}$p<0.01 vs control.

2.3.4. Effect of NaHS on 8B-cAMP-augmented $[Ca^{2+}]_i$ transients and contraction in electrically-stimulated ventricular myocytes

To delineate whether the effect of H2S is via altering cAMP/PKA pathway, 8B-cAMP (a cellular membrane-permeable cAMP analog) was employed. As shown in Figure 2-4A and 2-4C, 8B-cAMP at 0.5 mM also obviously increased both electrically-stimulated $[Ca^{2+}]_i$ transients and twitch amplitude. However, NaHS failed to attenuate both the parameters enhanced by 8B-cAMP ($\Delta[Ca^{2+}]_i$ transients amplitude: 8B-cAMP: 0.23 ± 0.04 NaHS+8B-cAMP group: 0.23 ± 0.06; n = 6, p = 0.87; Δtwitch amplitude: 8B-cAMP: 49 ± 10%; NaHS+8B-cAMP group: 49 ± 6%; n = 5, p = 0.99, Figure 2-4E). Figure 2-4F and 2-4G show that H_2S did not alter the effect of 8B-cAMP on both ±dl/dt and EI-$[Ca^{2+}]_i$ decay. The calcium response kinetics of 8B-cAMP again showed no difference between the groups in the presence or absence of NaHS (Figure 2-4H). These data suggest that the action site of H_2S may be upstream of PKA activation in β-adrenergic system.



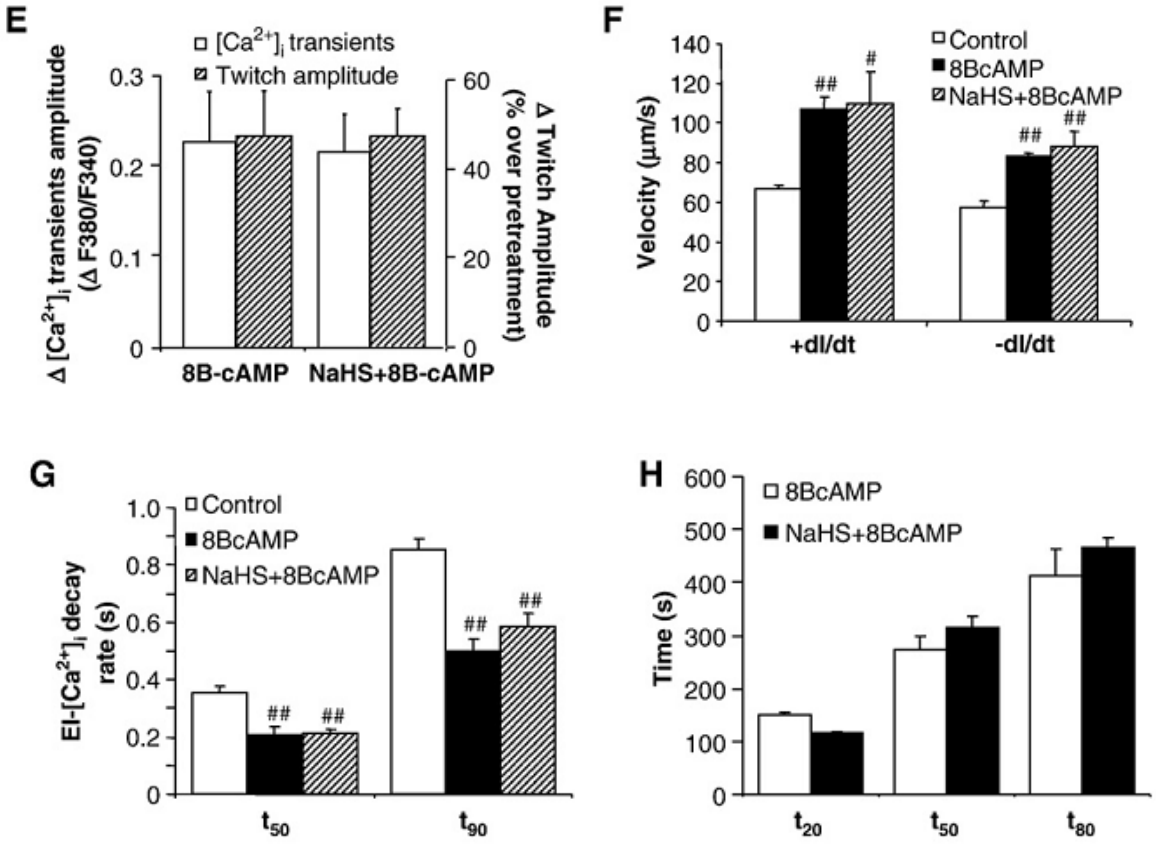
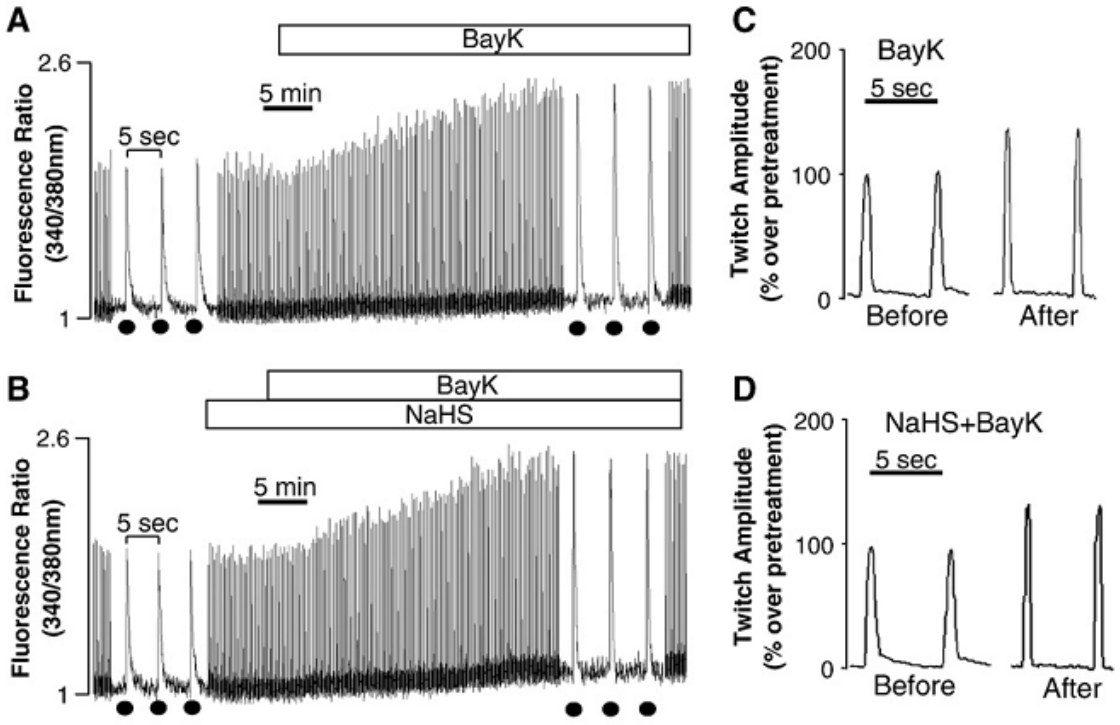


Figure 2-4 NaHS failed to alter the effects of 8B-cAMP on $[Ca^{2+}]_i$ transients and twitch amplitude in the electrically-stimulated ventricular myocytes. (A-D) Representative tracings showing the effect of 0.5 mM 8B-cAMP on electrically-induced $[Ca^{2+}]_i$ transients (A & B) and twitch amplitude (C & D) in the ventricular myocytes in the absence (A & C) and presence (B & D) of 100 μM NaHS. The time scale for time-expanded section and compressed section in $[Ca^{2+}]_i$ transient tracings are indicated separately. • Electrical stimulation, each • is separated by 5 sec. (E-H) Group data showing the effect of NaHS (100 μM) on 8B-cAMP-augmented amplitudes of electrically-induced $[Ca^{2+}]_i$ transients and myocyte twitch (E), maximal velocities of cell shortening and relaxing (F), decay rate of EI-$[Ca^{2+}]_i$ transients (G) and calcium response kinetics (H). Mean±S.E.M, n = 5-6, $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs control.

2.3.5. Effect of NaHS on Bay K-8644-augmented $[Ca^{2+}]_i$ transients and contraction in electrically-stimulated ventricular myocytes

Bay K-8644 (BayK) is a specific L-type Ca^{2+} channel agonist. I found that BayK (0.5 μM) significantly increased both amplitudes of $[Ca^{2+}]_i$ transients and myocyte twitch in electrically-stimulated ventricular myocytes (Figure 2-5A & 2-5C). These effects was not affected by H_2S ($\Delta[Ca^{2+}]_i$ transients amplitude: BayK group: 0.23 ± 0.05; NaHS+BayK group: 0.25 ± 0.09; p = 0.64, n = 5; Δtwitch amplitude: BayK group: 39 ± 5.2%; NaHS+BayK group: 29 ± 2%; p = 0.10, n = 5; Figure 2-5E). There was also no significant difference in ±dl/dt and decay rate of EI-[$Ca^{2+}]_i$ and calcium response kinetics between the groups with or without NaHS treatment (Figure 2-5F-H). These data exclude the possibility that H_2S may directly act on L-type calcium channels.



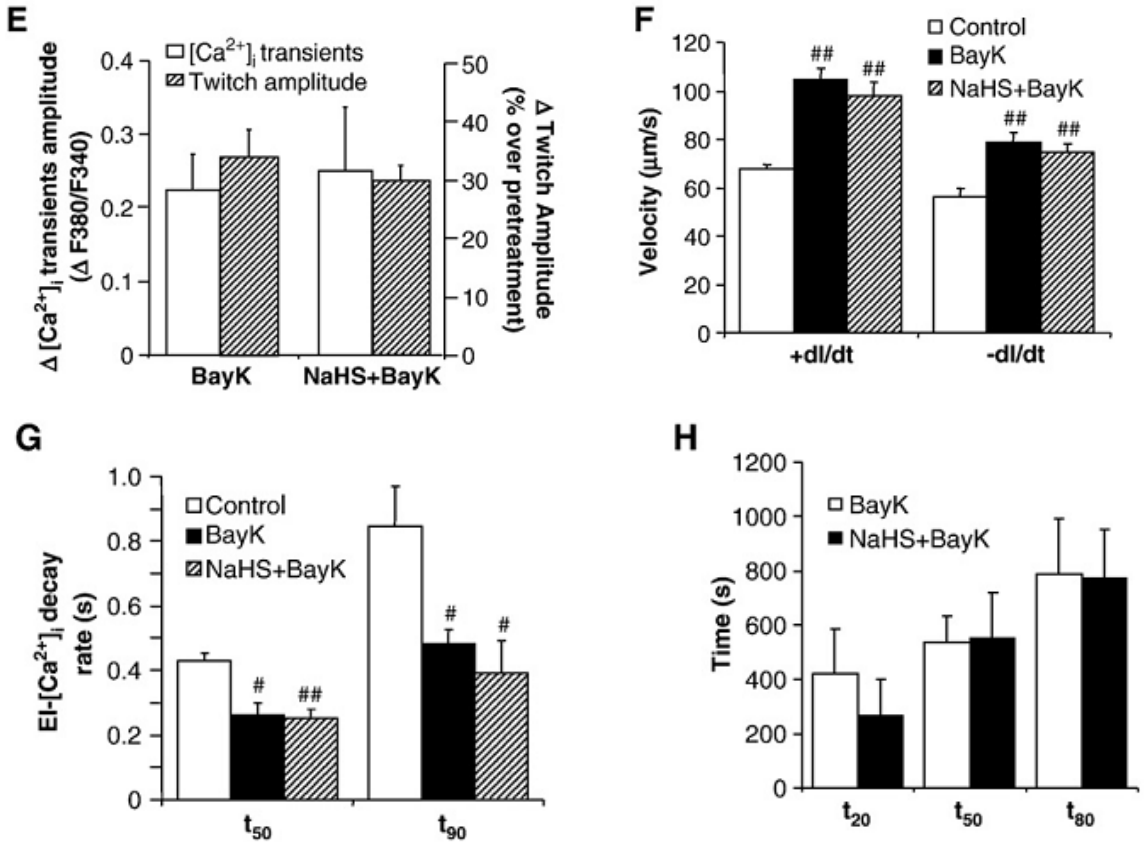


Figure 2-5 NaHS failed to alter the effects of BayK on $[Ca^{2+}]_i$ transients and twitch amplitude in the electrically-stimulated ventricular myocytes. (A-D) Representative tracings showing the effect of 0.5 μM BayK on electrically-induced $[Ca^{2+}]_i$ transients (A & B) and twitch amplitude (C & D) in the ventricular myocytes in the absence (A & C) and presence (B & D) of 100 μM NaHS. The time scale for time-expanded section and compressed section in $[Ca^{2+}]_i$ transient tracings are indicated separately. • Electrical stimulation, each • is separated by 5 sec. (E-H) Group data showing the effect of NaHS (100 μM) on BayK-augmented amplitudes of electrically-induced $[Ca^{2+}]_i$ transients and myocyte twitch (E), maximal velocities of cell shortening and relaxing (F), decay rate of EI-$[Ca^{2+}]_i$ transients (G) and calcium response kinetics (H). Mean±S.E.M, n = 5, $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs control.

2.3.6. Effect of NaHS on the elevated production of cAMP by ISO in rat ventricular myocytes

To further determine whether a cAMP-dependent pathway was involved in the interaction between H_2S and β-adrenergic receptor, I studied the effects of H_2S on ISO-stimulated and basal cAMP production in the rat ventricular myocytes. As shown in figure 2-6A, ISO (0.5 μM) significantly elevated the intracellular cAMP level, which was markedly attenuated by 100 μM NaHS, which alone did not significantly altered cAMP level. (Control group: 5.4 ± 0.4 pmol/mg protein; NaHS alone: 3.8 ± 0.5 pmol/mg protein; ISO group: 9.6 ± 1.0 pmol/mg protein; NaHS+ISO group: 6.2 ± 0.7 pmol/mg protein; n = 8-10, $p < 0.01$ for both Control vs ISO and ISO vs NaHS+ISO; Figure 2-6A).

2.3.7. Effect of NaHS on adenylyl cyclase activity in isolated rat hearts

I further examined whether the decreased cAMP production by NaHS is caused by inhibition of adenylyl cyclase. As shown in Figure 2-6B, forskolin (100 μM) stimulated adenylyl cyclase activity from 582 ± 85 pmol cAMP/mg protein/min to 1103 ± 139 pmol cAMP/mg protein/min (n = 11, $p<0.01$). NaHS at 100 μM significantly attenuated this effect (685 ± 106 pmol cAMP/mg protein/min; n = 11, $p<0.05$), suggesting that the effect of NaHS is due to the inhibition of AC activity.

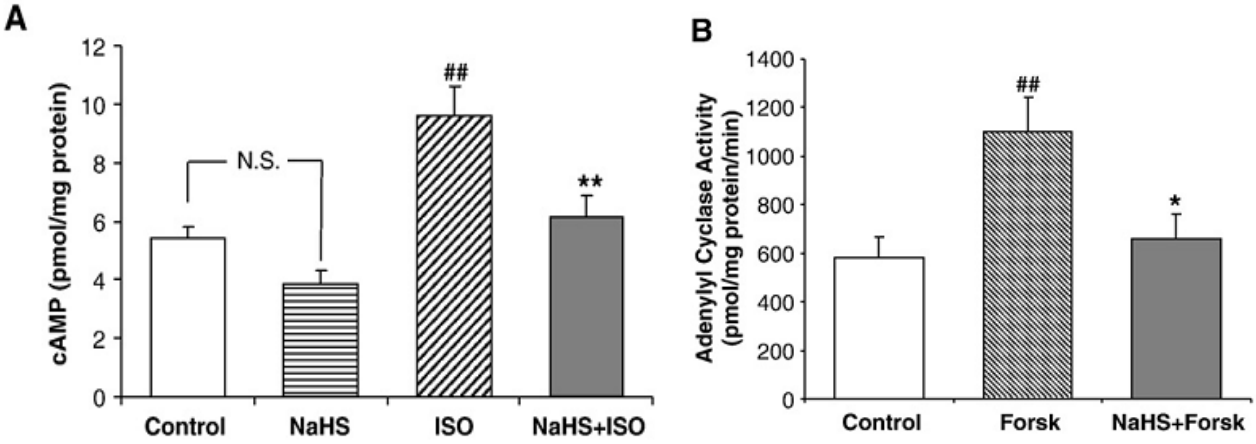


Figure 2-6 Effect of NaHS on cAMP production and AC activity in rat isolated cardiomyocytes or isolated hearts. (A) NaHS (100 μM) lowered intracellular cAMP stimulated by ISO in rat ventricular myocytes. Mean±S.E.M, n = 8-10, $^{\#\#}p<0.01$ vs control; **$p<0.01$ vs ISO. (B) NaHS (100 μM) inhibited AC activity stimulated by forskolin in isolated hearts. Mean±S.E.M, n = 9-11 $^{\#\#}p<0.01$ vs control, *$p<0.01$ vs forskolin group.

2.3.8. Effect of β-adrenergic stimulation on the production of H_2S in rat ventricular myocytes

Since β-adrenoceptor is over-stimulated during ischemia(Schomig and Richardt, 1990), I further examined whether β-adrenergic over-stimulation contributes to the lowered endogenous H_2S production in ischemia. The isolated cardiac myocytes were incubated with 10 μM ISO for 3 hours. As shown in Figure 2-7, ISO significantly decreased H_2S production in rat ventricular myocytes by 55±5.5% (n = 4, $p<0.01$). This result suggests that over-stimulation of β-AR might contribute to the lowered H_2S production during ischemia.

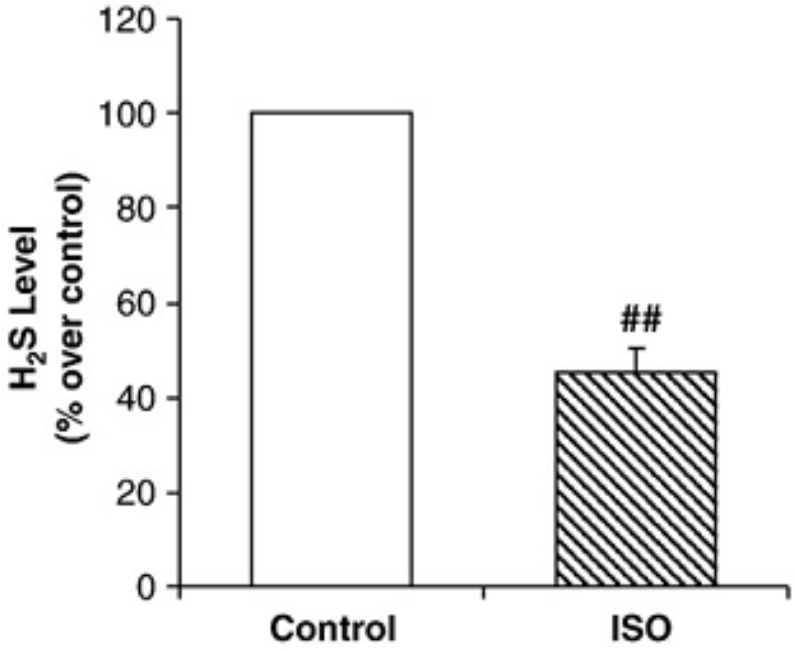


Figure 2-7 Effect of ISO on H_2S production in rat ventricular myocytes. Treatment with ISO (10 μmol/L) for 3 hours significantly decreased H_2S production in rat ventricular myocytes. $^{\#\#}p<0.01$ vs control, n = 4.

2.4. Discussion

The β-adrenergic receptor (AR) is the predominant post synaptic receptor to the sympathetic nervous innervations in the heart. It is conveniently categorized into 2 distinct subtypes, β1 and β2 receptors. Isoproterenol (ISO), a non-selective β-AR agonist, acts on both β1 and β2 receptors. It has been recognized that β1-AR is the predominant AR expressed by cardiomyocytes and β2-AR–dependent signals may represent only a relatively minor component of catecholamine responsiveness under normal physiological conditions (Molenaar and Parsonage, 2005). For this reason, the results presented in this study may indicate, to a greater extent, the effect of H_2S on the β1-adrenergic system signaling pathway. ISO has a stronger affinity to β-AR than that of norepinephrine, and the results obtained from the experiments using ISO are generally more physiological relevant than that using selective agonist for β1 receptor. In the present study, I found that H_2S significantly attenuated the amplitudes of both electrically-induced $[Ca^{2+}]_i$ transients and myocyte twitch upon β-adrenergic stimulation. The effect of H_2S is dependent on the action of ISO. H_2S only attenuated myocyte contraction stimulated by ISO ranged from $5x10^{-9}$ to 10^{-6} M, but not at lower concentrations (10^{-10}~10^{-9} M). Since ISO at 10^{-10}-10^{-9} M does not significantly increase cAMP levels (Vila Petroff et al., 2001), our data suggest that H_2S may negatively regulate β-AR when it is activated.

Activation of β-AR stimulates Gs-protein/adenylyl cyclase, which in turn increases intracellular cAMP level and therefore activates PKA. Activation of PKA phosphorylates key components of the calcium handling and contractile machinery. I

then studied the action mechanism of H_2S by stimulating different enzymes/channel proteins in the signaling cascade of β-AR system. I found that H_2S significantly attenuated the effects of ISO and forskolin, but failed to alter the effects of 8B-cAMP and BayK on amplitudes and velocities of myocyte contraction/relaxation and amplitudes of EI-[$Ca^{2+}]_i$ transients. To further confirm the exact action site of H_2S on β-adrenergic system, I examined the effect of NaHS on intracellular cAMP level and AC activity. It was found that NaHS markedly attenuated ISO-elevated intracellular cAMP and forskolin-stimulated AC activity, indicating that H_2S may negatively regulate β-AR function through inhibition of cAMP/PKA pathway. Since the ISO-induced activation of PKA phosphorylates and opens L-type calcium channels in the plasma membrane, H_2S may therefore attenuate ISO augmented amplitudes of EI-[$Ca^{2+}]_i$ transients and myocyte contraction.

Activation of PKA may also phosphorylate phospholamban (Simmerman and Jones, 1998) which, in turn, stimulates Ca^{2+} uptake through SERCA. Since I found that NaHS inhibited cAMP/PKA pathway, the up-regulated SERCA activity by ISO should also logically be inhibited by NaHS. However, I failed to see this response in this study. This may be related to other indirect effects of H_2S. I previously reported that H_2S preconditioning activates PKC which may, in turn, enhance SR-Ca^{2+} uptake (Pan et al., 2007). This stimulatory effect via PKC may compensate/counteract the inhibitory effect of H_2S on SERCA via suppressing cAMP/PKA pathway. This therefore causes the overall effect that H_2S failed to change SERCA function as observed in the present study.

In addition to cAMP/PKA pathway, a recent study demonstrated that a novel pathway downstream of β-AR involving the cAMP-dependent Rap GTP exchange factor (Epac) and PLCε regulates Ca^{2+}-induced Ca^{2+} release (CICR) in cardiac myocytes (Oestreich et al., 2007). It was found that, cAMP-dependent activation of Epac is also required for maximal β-AR stimulation of CICR in the myocardium. Smrcka et al indicated that 50-60% of β-AR dependent increase in CICR is attributed to PKA with the remaining 40-50% attributed to Epac-Rap-PLC pathway.(Smrcka et al., 2007) These studies further highlight the effectiveness of H_2S as a regulator of myocyte contractility since here I reported that H_2S may inhibit both cAMP related pathways.

H_2S has also been shown to open the K_{ATP} channels in vascular smooth muscle cells (Wang, 2002) and cardiac myocytes (Bian et al., 2006; Pan et al., 2006). Opening of the K_{ATP} channel generates an outward current and changes the membrane potential and causes hyperpolarisation, which may in turn inhibit the calcium influx via L-type Ca^{2+} channel and prevent Ca^{2+} overload. However, the involvement of the K_{ATP} channel in this H_2S-mediated-negative modulation of β-adrenergic stimulation may be insignificant due to the observations that H_2S did not affect the actions of 8B-cAMP and BayK on the $[Ca^{2+}]_i$ transients and contraction augmentation. This is supported by the findings from several groups. Firstly, ISO was shown to be able to open K_{ATP} channel in coronary smooth muscle cell through stimulation of cAMP/PKA pathway (Wellman et al., 1998). Thus, ISO may also activate K_{ATP} channel in cardiac myocytes by the same pathway. For this reason, H_2S may not exert any significant regulative effect on β-adrenergic stimulation through opening of the K_{ATP} channel. Secondly, it has been reported that myocytes K_{ATP} channels play a negligible role in modulating intact in vivo

cardiac contraction or arrhythmia in normal and failing heart with and without increased metabolic demand and regional ischemia (Saavedra et al., 2002). These data support our findings that H_2S negatively regulated β-adrenergic function is via inhibition of cAMP production but not via activation of K_{ATP} channels.

In myocardial ischemia, sympathetic activity in the heart is closely linked with the progression of cell injury and the incidence of ventricular arrhythmias (Schomig, 1990). Excess norepinephrine release and accumulation in hearts may induce number of physiological processes including depletion of ATP and accumulation of intracellular Ca^{2+} and Na^{+} (Cascio et al., 2005), all of which play important roles in the genesis of ventricular arrhythmias (Omerovic et al., 2007). Therefore, H_2S may antagonize negative consequences of sympathetic overactivation during ischemia by generating negative feedback to cAMP production. In this context, H_2S replacement therapy may be a significant cardioprotective and anti-arrhythmic intervention for those patients with chronic ischemic heart disease whose plasma H_2S level was found to be reduced. (Jiang et al., 2005)

A clinical observational study has shown that the plasma concentration of H_2S in patients with coronary heart disease was significantly lowered in comparison with that in normal control subjects, suggesting that decreased plasma H_2S levels may correlate with the severity of coronary heart disease (Jiang et al., 2005). In the present study, I showed that over-stimulation of β-AR by ISO reduced the endogenous production of H_2S. This is consistent with our previous report that ischemia decreases endogenous H_2S production in ventricular myocytes (Bian et al., 2006). In addition, Geng et al has also showed that reduced myocardial and plasma H_2S levels were found in the rats

injected with ISO.(Geng et al., 2004a) These reports strongly imply that reduced H_2S level caused by ischemia and β-adrenoceptor over-stimulation may result in the impairment of the negative modulation of H_2S on β-adrenoceptor system, and hence calcium overload. Our findings may provide a novel mechanism of ischemia-induced myocardial injury and arrhythmia.

The mechanisms of ISO-induced disturbance of the CSE/H_2S pathway are unclear. CSE is a key enzyme for H_2S production in heart and its activity depends on pyridoxal 5'-phosphate (PLP), which is reduced in ischemic disease such as stroke (Kelly et al., 2004). Thus, ISO may mimic the ischemic condition and result in lowered PLP levels, which might inhibit the CSE activity and cause decrease in H_2S generation. In neonatal cardiac myocytes, it has been reported that ISO may elicit transient release of NO (Anuar et al., 2006), which has been shown to down-regulate CSE mRNA level (Kanai et al., 1997). Therefore, the inhibited biosynthesis of H_2S by ISO may also via altering NO release.

In conclusion, the present study has demonstrated for the first time that exogenous application of H_2S at biological-relevant concentration negatively regulates β-adrenergic function via inhibition of adenylyl cyclase. Sympathetic regulation of cardiac function is important for the normal response of the heart to stress. Perturbation of β-adrenergic signaling significantly contributes to progressive heart failure. Our study therefore not only demonstrates a significant cardioprotective intervention, but may also shed some light on understanding the pathogenesis of ischemic heart disease.

Chapter 3 Role of Hydrogen Sulfide in the Cardioprotection Induced by Ischemic Preconditioning

3.1. Introduction

In the previous chapter, it was found that H_2S negatively regulates beta-adrenergic function, which suggests that H_2S may be cardioprotective against ischemia/reperfusion injury, when the beta adrenergic system is over-stimulated (Schomig et al., 1984). As such, in this chapter, I continued to study whether preconditioning with H_2S can protect the heart against ischemic injury and the contribution of H_2S in the cardioprotection caused by ischemic preconditioning (IP).

Ischemic preconditioning (IP) refers to the phenomenon that previous exposure to brief sublethal ischemia provides protection on the heart against subsequent severe ischemia insults (Murry et al., 1986). It is a powerful natural cardioprotective mechanism. A pivotal signaling event during IP is the opening of K_{ATP} channels (Sanada and Kitakaze, 2004). The ability of H_2S to open K_{ATP} channels in smooth muscle cells (Zhao et al., 2001) prompted us to investigate whether endogenous H_2S also plays a part in the IP-induced cardioprotection.

3.2. Materials and methods

3.2.1. Assessment of cell viability and morphology

Trypan blue exclusion was used as an index of myocyte viability (Zhou et al., 1996; Hiebert and Ping, 1997). After cells were incubated with 0.4% (w/v) trypan blue dye for 3 min, living cells were unstained and termed nonblue cells. Nonblue cells/total cells were determined in a hemocytometer chamber using a light microscope (10x magnification). Cell morphology was also assessed by microscopic examination.

Percentage of rod-shaped (length/width ratio, >3:1) cells were determined as an indicator of the percentage of healthy cardiomyocytes. 200-500 cells in each of 5-7 cultures were tested for each group. Percentage of rod-shaped (length/width ratio, >3:1) cells were determined as an indicator of the percentage of healthy cardiomyocytes. 200-500 cells in each of 5-7 cultures were tested for each group.

In *in vitro* myocytes study, the ischemic solution (pH 6.6 containing 2 x 10-2 mol/L lactate and 10-2 mol/L 2-deoxy-D-glucose (2-DOG), an inhibitor of glycolysis) was used according the protocol shown in Figure 3-2 A, to mimic the ischemic condition in the heart.

3.2.2. Statistical Analysis

Values presented are mean ± standard error of mean. SPSS for Windows (11.5.0, LEAD technologies, USA) was used to perform the analysis. One-way analysis of variance (ANOVA) was used to determine the difference among groups. A post hoc Bonferroni analysis was used to determine the significant differences between the groups. The significance level was set at $P<0.05$

3.2.3. Isolated Perfused Rat Heart Preparation

Sprague-Dawley rats (230-270 g, male) were anesthetized with 200 mg/kg pentobarbitone by intraperitoneal (i.p.) injection. Heparin (1000 IU) was administered i.p. to prevent coagulation during removal of the heart. The heart was removed, mounted in a Langendorff apparatus and perfused retrogradely through the aorta with a Krebs solution containing (mM; 117 NaCl, 5 KCl, 1.2 MgSO4, 1.2 KH_2PO_4, 1.25 $CaCl_2$, 25 $NaHCO_3$ and 11 glucose) and bubbled with 95% O_2/5% CO_2 (pH 7.4, 34 ^oC), at a constant flow rate of 13 ml/min as described previously (Bian et al., 2000). The

Electrocardiogram (ECG) was monitored continuously and recorded with two electrodes hooked to the apex and the aorta respectively. Each heart was allowed to stabilize for 15 min before the experiment commenced. Any heart that exhibited arrhythmias during this period was discarded.

The experimental design for the sodium hydrogen sulfide preconditioning (SP) and IP protocols is shown in Figure 1A. In the vehicle preconditioning (VP) group, hearts were superfused for 40 min with Krebs solution (13 ml/min) and then subjected to a low flow ischemia insult (perfusion rate, 0.5 ml/min, 30 min) followed by 10 min of reperfusion. For the preconditioning group, hearts were subjected to 3 cycles of 3 min of perfusion with sodium hydrogen sulfide (NaHS) (100 μM, SP) or low flow ischemia insults (perfusion rate 0.5 ml/min, IP) separated by 5 min of superfusion with normal Krebs solution at normal perfusion rate (13 ml/min). After SP or IP, hearts were subjected to low flow ischemia insults for 30 min followed by 10 min reperfusion as in the VP group. In an attempt to determine the involvement of H_2S formation in the cardioprotection induced by IP, either DL-propargylglycine (PAG, 2 mM) or β-cyano-L-alanine (BCA, 1 mM) (both CSE inhibitors) were given 15 min before as well as during IP (Figure 1A). The concentration of such drug chosen was based on a previous study.(Mok et al., 2004)

3.2.4. Arrhythmia Scoring System

In order to quantify arrhythmias, the scoring system of Curtis and Walker (Curtis and Walker, 1988) was used with modifications. Since the arrhythmias induced by ischemia/reperfusion in the present study were mainly ventricular premature beats and ventricular tachycardia (VT), scoring emphasis was placed on ventricular arrhythmias.

The scoring system adopted was therefore as follows: 0: no arrhythmia; 1: 1-30 premature ventricular contraction (PVC); 2: >30 PVC; 3: <3 episode of ventricular fibrillation (VF)/ VT; 4: 3-5 episode of VF/VT; 5: >5 episode of VF/VT. The score of a particular heart was the value of the most severe type of arrhythmias exhibited during of 10 min reperfusion.

3.2.5. Other methods

Isolation of cardiomyocytes and intracellular Ca^{2+} imaging have been described in the Materials and Methods in Chapter 2.

3.2.6. Drugs and chemicals

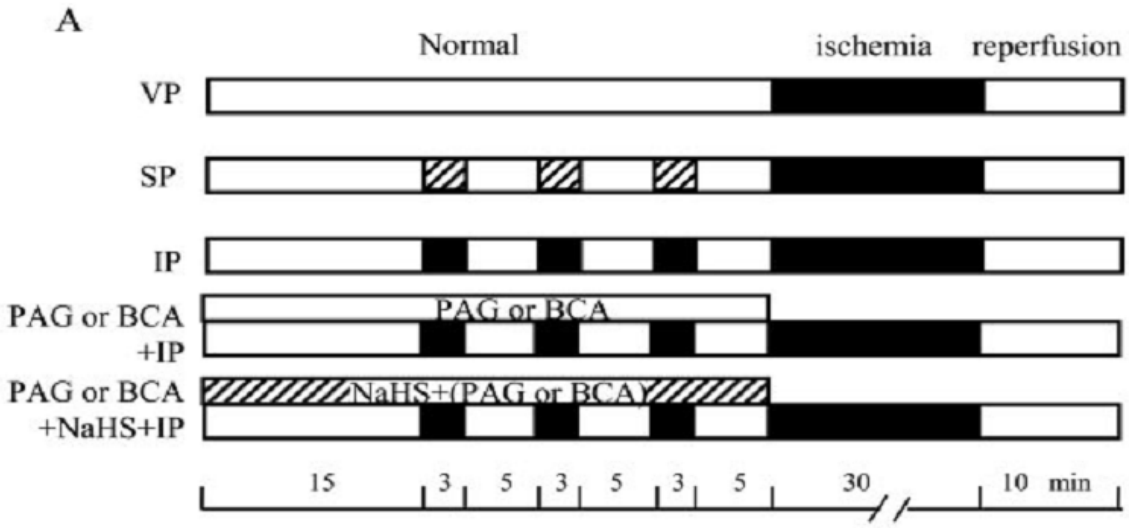
Type 1 collagenase, protease XIV, 2-DOG, PAG, BCA, NaHS, 5-HD, and N, N-dimethyl-p-phenylenediamine sulphate, FeCl3 and trypan blue dye were purchased from Sigma Chemical Co, USA. Glibenclamide was obtained from Tocris Cookson Ltd, UK. HMR-1098 was a generous chased from Molecular Probes Inc. USA. Chelerythrine and BSM were from Calbiogift from Aventis Pharma Deutschland GmbH (Frankfurt, Germany). Fluo-3 was purchem. All chemicals were dissolved in distilled water except Fluo-3/AM, chelerythrine and BSM, which were dissolved in DMSO at a final concentration <0.1% (w/v).

3.3. Results

3.3.1. NaHS preconditioning (SP) attenuated ischemia/reperfusion-induced arrhythmias

To determine whether SP is able to produce a cardioprotective effect on cardiac rhythm, I measured ECG in isolated rat hearts. Like numerous previous researchers, I utilized NaHS as a soluble H_2S donor drug. Previous studies have shown that the actual amount of H_2S generated in such solutions is about 33% of the amount of NaHS (Reiffenstein et al., 1992). As such NaHS (100 μM) is likely to produce approximately 33 μM H_2S, which is well within the physiological concentration range in, for example, rat plasma (Zhao et al., 2001).

Figure 3-1B & 3-1C show that low-flow ischemia/reperfusion induced severe arrhythmias in the vehicle preconditioning (VP) group. Both the duration of arrhythmias (VP:78.2±19.6 s vs SP:8.8±4.7 s, n=6, P<0.01) and the arrhythmia scores (VP:3.6±0.2 vs SP: 0.5±0.4, n=6, p<0.001) during the 10 min reperfusion period were significantly decreased in the SP group. These data suggest that SP protects the heart against ischemia/reperfusion induced arrhythmias.



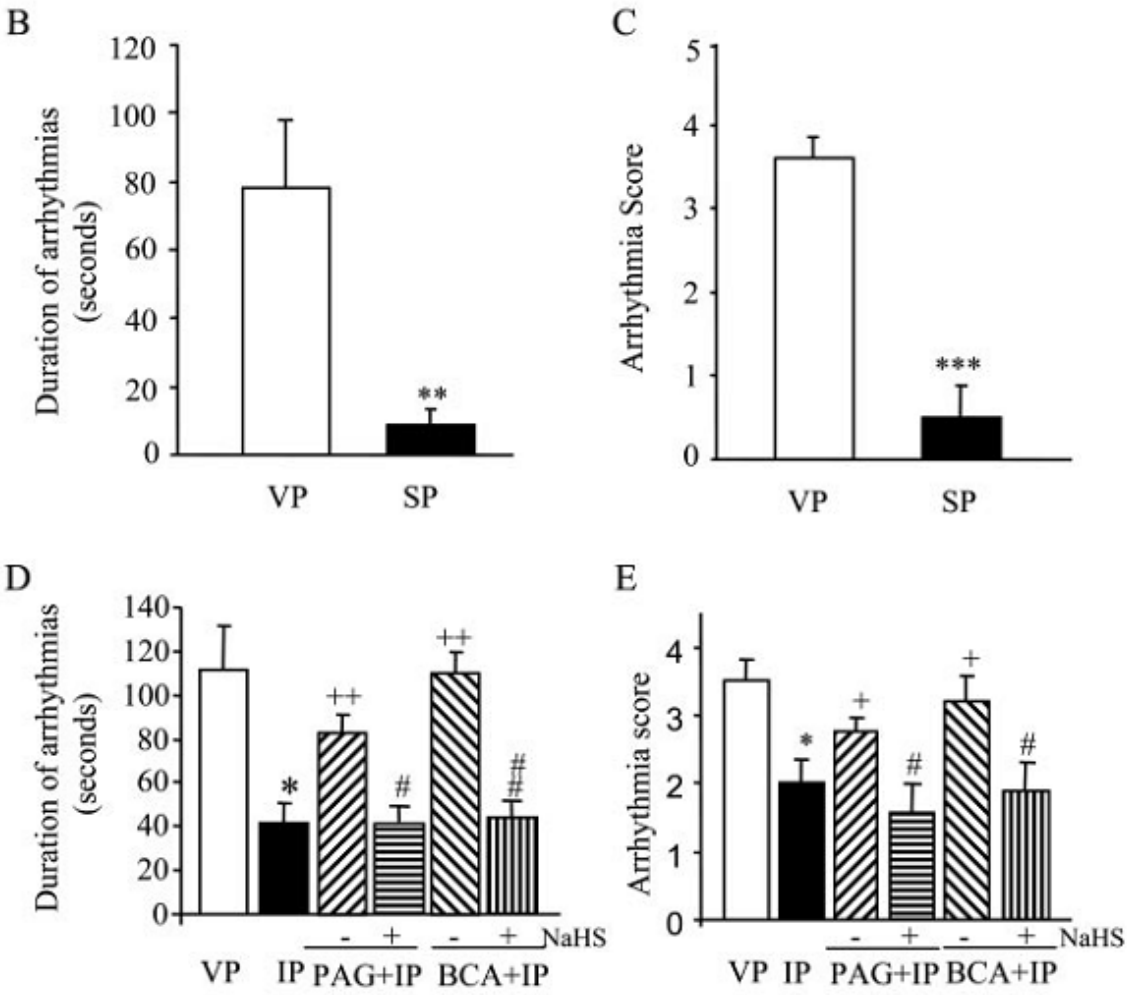


Figure 3-1. Effect of (NaHS preconditioning) SP and (Ischemic preconditioning) IP on cardiac rhythm in the isolated perfused rat heart during ischemia/reperfusion. A, Experimental design. Solid fields: low flow perfusion with Krebs solution at a perfusion rate of 0.5 ml/min; open fields: Krebs solution at perfusion rate of 13 ml/min; slashed fields: Krebs solution with 100 μM NaHS. 2 mM PAG or 1 mM BCA was administered with/without NaHS 15 min before and during 3 cycles of IP. B & C, SP significantly decreased the duration of arrhythmias (B) and arrhythmia scores (C) during reperfusion compared to those in the control group. Mean±SEM; n=6, $^{**}P<0.01$; $^{***}P<0.001$ *vs* VP. D & E, Role of endogenous H_2S in the cardioprotection of IP on ischemia/reperfusion-induced arrhythmias. Co-administration of NaHS reversed the effects of PAG and BCA. Mean±SEM; n=5-8, $^{*}P<0.05$ *vs* VP, $^{+}P<0.05$, $^{++}P<0.01$ *vs* IP, $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs* PAG+IP or BCA+IP.

3.3.2. Effect of SP on cell viability and morphology subjected to ischemia solution

To further substantiate the cardioprotective effect of H_2S, I also assessed the concentration-dependent effect of NaHS on cell viability and morphology in isolated rat ventricular myocytes which were exposed to ischemia solution. As shown in Figure 3-2B, preconditioned with 3 cycles of different concentrations of NaHS (1 μM, 10 μM, 100 μM and 1 mM), the percentage of non-blue cells following ischemia increased in a concentration dependent manner. This effect was significantly greater than that of the VP group at an NaHS concentration of 10 μM and the maximum protective response was observed at a concentration of 100 μM NaHS (VP: 32.6±2.1%, 10 μM NaHS: 45.9±2.3%, 100 μM NaHS: 47.9±2.2%, all n=7; Figure 3-2B).

To compare the responses in terms of myocyte viability and cell shape, the rod-shaped cells were counted 10 min into the reperfusion period. As shown in Figure 3-2C, treatment with NaHS at 10 μM and 100 μM resulted in a greater percentage of rod-shaped cells per the total number of cells than that detected in the VP group (VP: 28.9±3.3%, 10 μM NaHS: 41.3±2.8%; 100 μM NaHS: 43.4±3.1%; all n=7, Figure 3-2C).

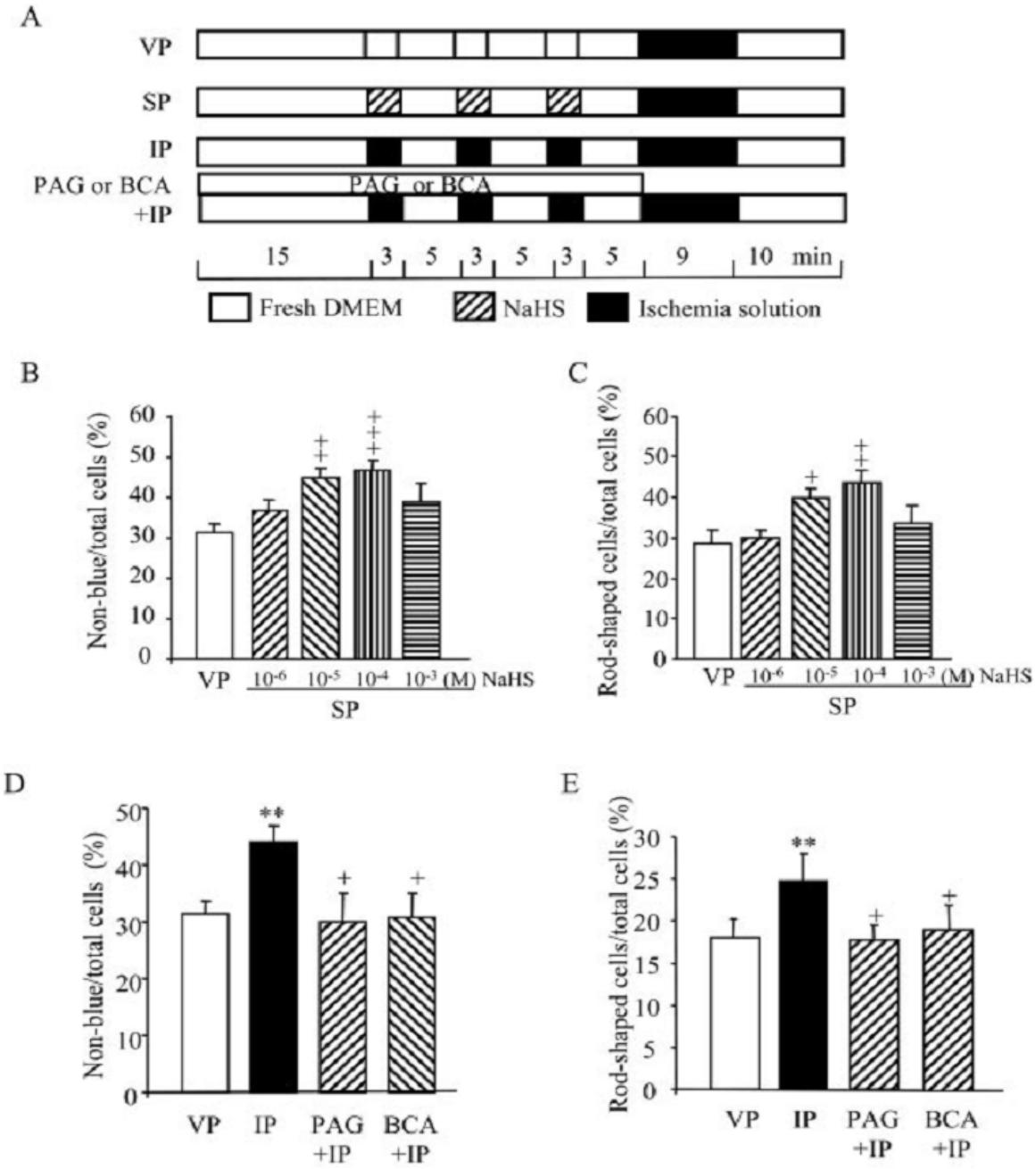


Figure 3-2. Effects of SP and IP on cell viability and morphology of ventricular myocytes. A, Experimental design. Solid fields: simulated ischemia solution (glucose-free Krebs solution containing 10 mM 2-DOG and 10 mM $Na_2S_2O_4$, pH6.6); open fields: fresh DMEM solution; slashed fields: DMEM solution containing different concentrations of NaHS. 2 mM PAG or 1 mM BCA was given 15 min before and during 3 cycles of IP. B, concentration-dependent effect of SP on cell viability. Non-blue cells are live cells. Mean±SEM; n=7 cultures of ≈500 cells each. $^{++}P<0.01$, $^{+++}P<0.001$ *vs* VP. C, Concentration-dependent effect of SP on cell morphology. Rod-shaped cells per total cells counted. Mean±SEM; n=7 cultures of ≈500 cells each. $^{+}P<0.05$, $^{++}P<0.01$ *vs* VP. D & E, Effects of IP on percentage of non-blue cells (D) and rod-shaped cells (E) in the presence and absence of PAG or BCA. Mean±SEM, n=6-18 cultures of ≈500 cells each. $^{**}P<0.01$ *vs* VP, $^{+}P<0.05$ *vs* IP.

3.3.3. Effect of SP on electrically-induced $[Ca^{2+}]_i$ transients of the ventricular myocytes subjected to ischemia solution.

To determine the functional status of the cells, electrically-induced $[Ca^{2+}]_i$ transients before, during and after ischemia were determined. As shown in Figure 3-3A and 3-3B, electrically-induced $[Ca^{2+}]_i$ transients were significantly ($p<0.001$) decreased after ischemia/reperfusion (25.8±3.0%, n=25). These results are in agreement with the effects of metabolic inhibition or hypoxia as reported in a previous study (Seki and MacLeod, 1995). Like the change in cell viability, the decrease in $[Ca^{2+}]_i$ transients during reperfusion were also significantly attenuated by SP (90.4± 4.6%; n=25; Figure 3-3A & 3-3B). These results suggest that cell function was significantly improved by SP.

3.3.4. Effects of IP on cardiac rhythm, cell viability and electrically-induced $[Ca^{2+}]_i$ transients in the presence and absence of H_2S synthase inhibitors

This series of experiments was designed to determine whether endogenous H_2S contributes to the cardioprotection of IP. PAG or BCA were administered as shown in Figure 3-1A. Perfusion with PAG or BCA alone for 40 min had no effect on cardiac rhythm (i.e. arrhythmia score and duration were 0). As shown in Figure 3-1D & 3-1E, IP significantly decreased the duration of cardiac arrhythmias (VP: 111.4±20.3, n=8; IP: 41.4±9.5; n=5, $p<0.05$; Figure 3-1D) and the arrhythmia scores (VP: 3.5±0.3, n=8; IP: 2.0±0.4, n=5; $p<0.05$; Figure 3-1E). Treatment with one of the two CSE inhibitors (2 mM

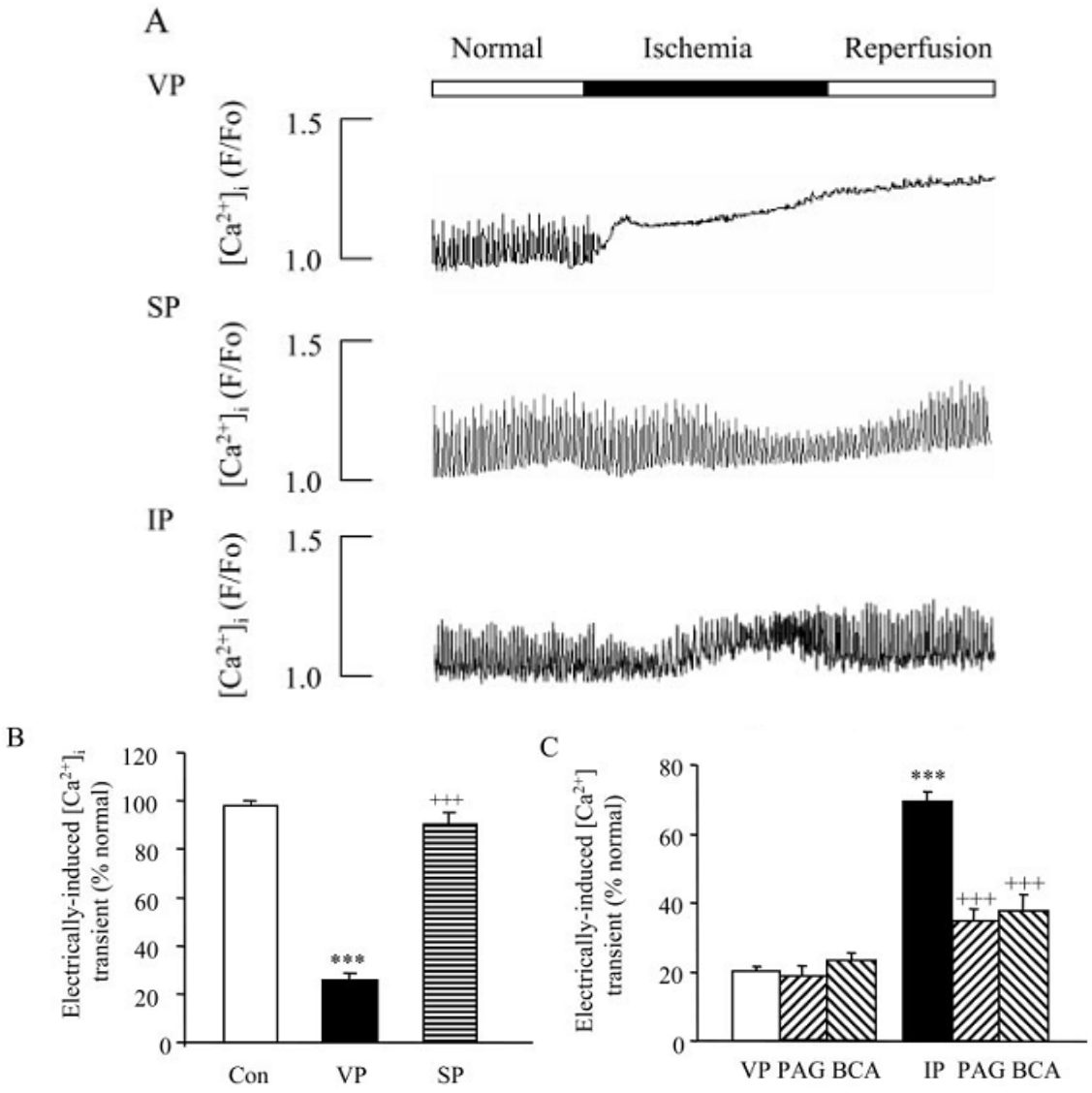


Figure 3-3. Effect of SP and IP on electrically-induced $[Ca^{2+}]_i$ transients in the single ventricular myocytes. Experimental procedures used were the same as those in Figure 2-2A. A, Representative tracings of electrically-induced $[Ca^{2+}]_i$ transients in the VP, SP and IP groups. B, Group results showing the amplitudes of electrically-induced $[Ca^{2+}]_i$ transients before and 10 min after reperfusion. Values are mean±SEM; n=25. ***P<0.001 *vs* the value in the control (Con) group; ^{+++}P<0.001 *vs* the value in the VP group. C, Effect of IP on electrically-induced $[Ca^{2+}]_i$ transients in the presence and absence of PAG or BCA. Mean±SEM, n=10-26, ***P<0.001 *vs* VP, ^{+++}P<0.001 *vs* IP.

PAG or 1 mM BCA (Teague et al, 2002) 15 min before and during IP) increased the duration of arrhythmias (PAG: 82.4±8.2, n=6; BCA:107.4±7.8, n=5; $p<0.05$; Figure 3-1D) and the arrhythmia scores (PAG: 2.75±0.2, n=6; BCA:3.2±0.4, n=5; $p<0.05$; Figure 3-1E) in the IP group. Co-administration of 100 μM NaHS abolished these effects, suggesting that the effects of PAG and BCA were most probably secondary to a decrease in endogenous H_2S. Taken together, these data suggest that myocyte endogenous H_2S production may be decreased during ischemia and IP may attenuate this effect.

To further confirm whether the cardioprotection associated with IP is mediated by endogenous H_2S, cell viability and electrically-induced $[Ca^{2+}]_i$ transients were used as markers. Neither PAG or BCA alone affected cell viability (Control: 69.2±3.2%, PAG: 63.4±2.4%, BCA: 65.1±1.7%, all n=5) or morphology (Control: 62.8±1.2%, PAG: 60.3±1.8%, BCA: 59.2±1.2%, all n=5). The percentage of non-blue cells and rod-shaped cells at 10 min into reperfusion in the IP group was significantly higher than that of the VP group (Figure 3-2D & 3-2E). Both PAG and BCA reversed the cardioprotection due to IP on cell viability and morphology. I also tested whether inhibition of endogenous H_2S formation with PAG or BCA reversed the cardioprotection of IP on cell function by observing the amplitude of electrically-induced $[Ca^{2+}]_i$ transients during 10 min of reperfusion. Figure 3-3A & 3-3C show that the amplitudes of electrically-induced $[Ca^{2+}]_i$ transients induced by IP were significantly higher than those in the VP group (VP: 20.2±3.8%; IP: 70.0±3.5%; n=26; $p<0.001$). Both PAG and BCA reversed the increased amplitudes in the IP group (PAG: 34.8±3.6%, n=19; BCA: 37.8±4.6%; n=10, $p<0.001$; Figure 3-3C), but by themselves

did not affect electrically-induced $[Ca^{2+}]_i$ transients in the VP group. Therefore, taken together, these data suggest that endogenous H_2S production is likely to contribute to the cardioprotection caused by IP.

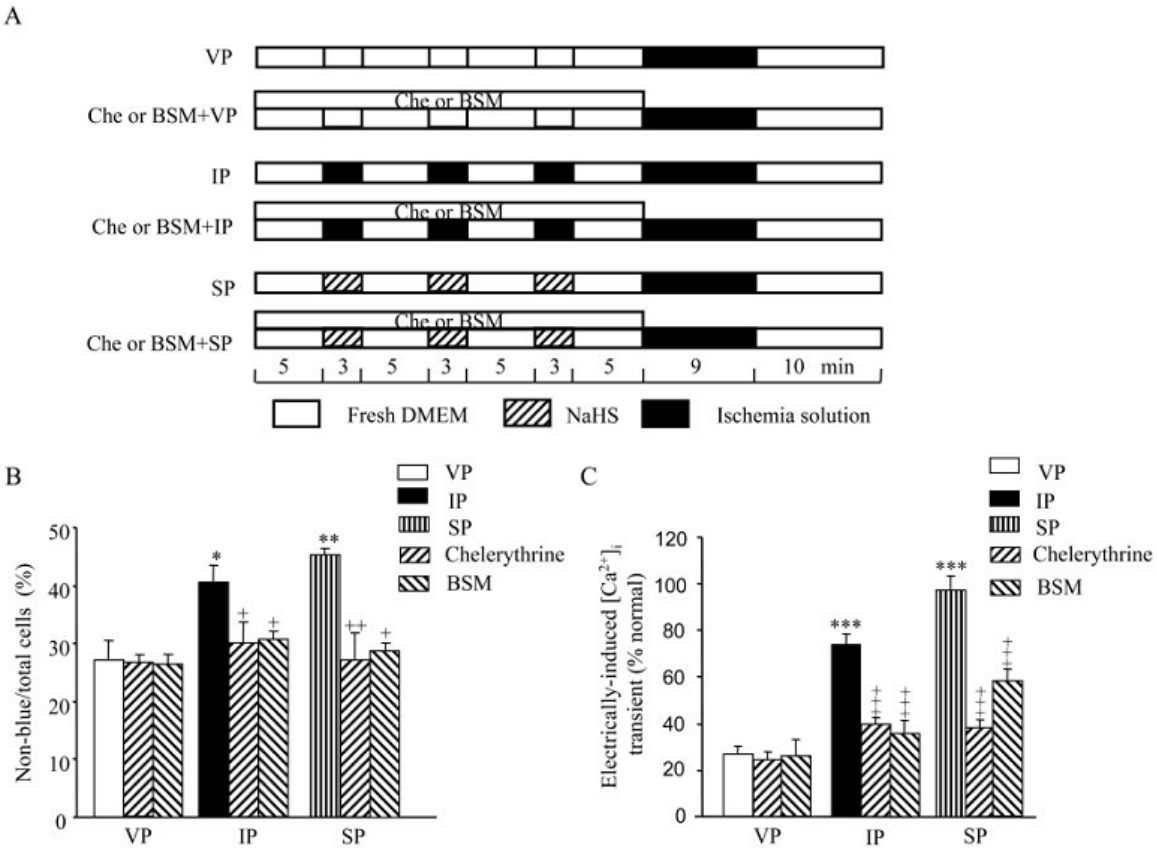


Figure 3-4. Effect of IP and SP on cell viability and electrically-induced $[Ca^{2+}]_i$ transients in rat ventricular myocytes in the presence and absence of PKC inhibitors. A, Experimental design. Chelerythrine (1 μM) or BSM (100 nM) was administrated 5 min before and during 3 cycles of IP, SP and VP. B, Group results of cell viability. Values are presented as non-blue cells per total myocytes counted. All values are mean±SEM; n=7 cultures of ≈500 cells each. **P*<0.05, ***P*<0.01 *vs* VP, ^{+}P<0.05 ^{++}P<0.01 *vs* IP or SP. C, Group results of the amplitudes of electrically–induced $[Ca^{2+}]_i$ transient. All values are mean±SEM; n=13-38. ****P*<0.001 *vs* VP, ^{+++}P<0.001 *vs* IP or SP.

3.3.5. Effects of IP and SP on cell viability and electrically induced $[Ca^{2+}]_i$ transients in the presence and absence of PKC inhibitors

The goal of this series of experiments was to probe the mechanism(s) involved in the cardioprotection conferred by IP and SP. To determine whether PKC is involved in cardioprotection induced by IP and SP, two specific PKC inhibitors, chelerythrine (1 μM) and BSM (100 nM) (Kawamura et al., 1998), were used. Either Chelerythrine or BSM were given 5 min before as well as during preconditioning. As shown in Figure 3-4B & 3-4C, chelerythrine or BSM alone had no significant effect on cell viability or function in the VP group. However, both drugs significantly reversed the cardioprotective effect of IP and SP on cell viability (IP: 40.5±3.0%; Chelerythrine: 30.1±3.6; BSM: 30.6±1.7; all n=7; $p<0.05$). Similarly, the improved cell functions in the IP and SP groups were also attenuated by the chelerythrine and BSM (Figure 3-4C). The amplitudes of electrically-induced $[Ca^{2+}]_i$ transients were decreased from 74.2±4.2% (n=26) in the IP group to 37.2±3.6% in the chelerythrine group (n=28, $p<0.001$) and 35.6 ±5.7% (n=13) in the BSM group and decreased from 97.3±5.8% (n=38) in the SP group to 38.0±3.3% in the chelerythrine group (n=25, $p<0.001$) and 58.2 ± 4.7% in the BSM group (n=18, $p<0.001$).

3.3.6. Effects of IP and SP on cell viability and electrically induced $[Ca^{2+}]_i$ transients in the presence and absence of K_{ATP} channel blockers

To determine the involvement of K_{ATP} channels in the cardioprotection induced by SP and IP, either glibenclamide (10 μM), a non-selective K_{ATP} channel blocker, 5-HD (100

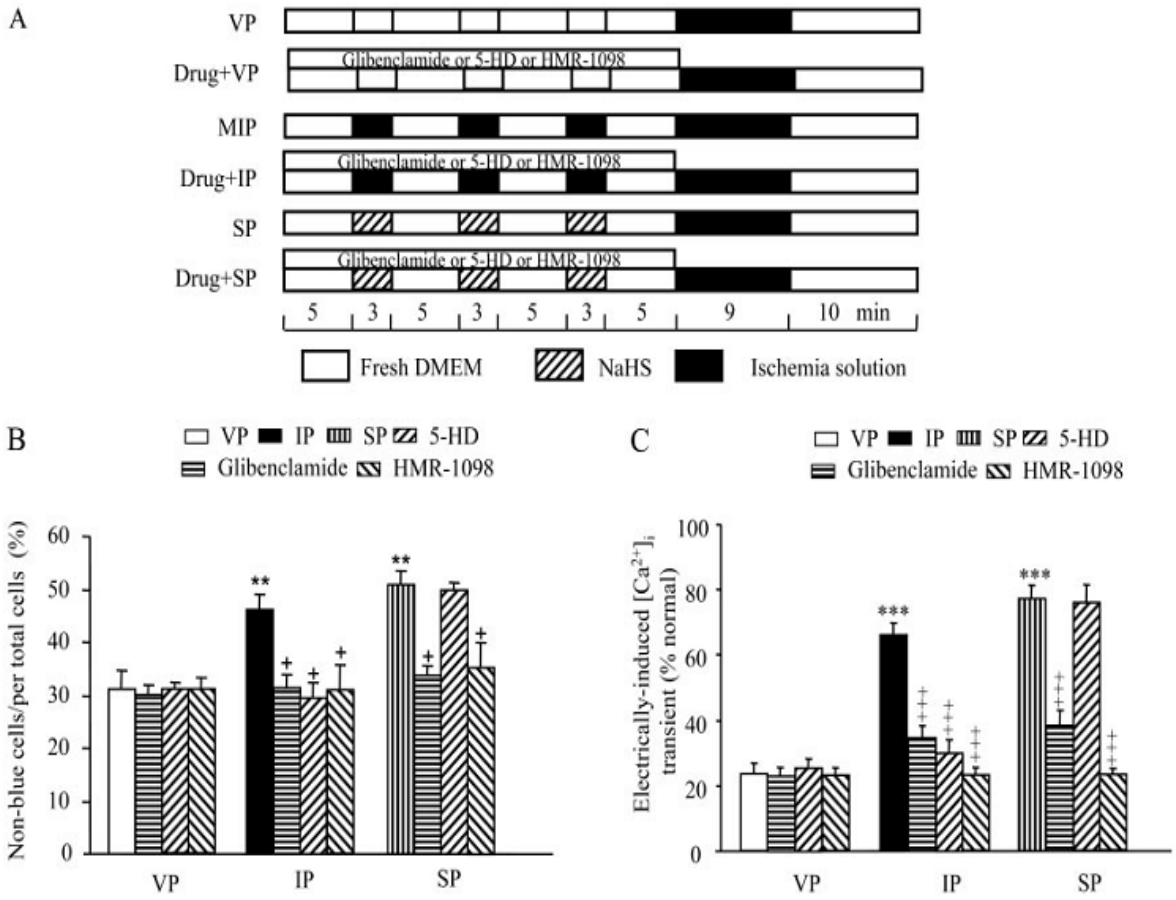


Figure 3-5. Effect of IP and SP on cell viability and electrically-induced $[Ca^{2+}]_i$ transients of rat ventricular myocytes in the presence and absence of K_{ATP} channel blockers. A. Experimental procedure. Glebenclamide, 5-HD and HMR-1098 were given 5 min before and during VP, SP or IP. B, Group results of cell viability. Values are presented as non-blue cells per total myocytes counted. Mean±SEM; n=8-18 cultures of ≈500 cells each. $^{**}P<0.01$ *vs* VP, $^{+}P<0.05$ *vs* IP or SP. C, Group results of the amplitudes of electrically–induced $[Ca^{2+}]_i$ transient. Mean±SEM; n=12-40, $^{***}P<0.001$ *vs* VP, $^{+++}P<0.001$ *vs* IP or SP.

μM), a selective mitoK_{ATP} channel blocker or HMR-1098 (30 μM), a selective sarcK_{ATP} blocker, were administered 5 min before and during preconditioning (Chen et al., 2003). The experimental procedures are shown in Figure 3-5A. As shown in Figure 3-5B & 3-5C, all three drugs alone did not affect cell viability and function in the VP group. Fig 3-5B shows that glibenclamide significantly reduced cell viability in both the IP and SP groups (IP: 44.9±3.1%, n=15; vs glibenclamide+IP: 32.2±3.7, n=9; $p<0.05$; SP:

50.8±2.7%, n=18; vs glibenclamide+SP: 34.1±2.7%; n=8; $p<0.05$). Similar results were also obtained in cell function using electrically-induced $[Ca^{2+}]_i$ transients as the end-point (Figure 3-5C). Glibenclamide significantly attenuated the increased amplitudes of electrically-induced $[Ca^{2+}]_i$ transients in the IP (IP: 67.9±3.5%, n=37; vs Glibenclamide+IP: 37.4±3.1%; n=40; $p<0.001$) and SP (SP: 81.9±3.7, n=12; vs Glibenclamide+SP: 39.4±4.1; n=32; $p<0.001$) groups. These data suggest that K_{ATP} channel is involved in the cardioprotection conferred by both IP and SP.

5-HD and HMR-1098 were further employed to determine the involvement of mitoK_{ATP} or sarcK_{ATP} channels in the cardioprotection of IP and SP. HMR-1098 significantly decreased the cell viability (IP+HMR: 30.9±4.6%; n=8; SP±HMR: 35.2±4.7%; n=8, $p<0.05$; Figure 3-5B) and the amplitudes of electrically-induced calcium transients (IP+HMR: 23.1±2.2%, n=21; SP+HMR: 23.0±3.3%%, n=12; Figure 3-5C) in both IP and SP group. However, 5-HD only significantly attenuated the cardioprotection of IP (cell viability: 29.6±2.8%, n=8; $[Ca^{2+}]_i$ transients: 30.1±4.2%, n=29), but had no significant effect on these parameters in the SP group (cell viability: 49.7±1.6%, n=8; $[Ca^{2+}]_i$ transients: 75.9±5.4, n=12, Figure 3-5A & 3-5B). These data suggest that unlike the mechanism of IP, sarcK_{ATP} channel, but not the mitoK_{ATP} channel, mediates the cardioprotection conferred by SP.

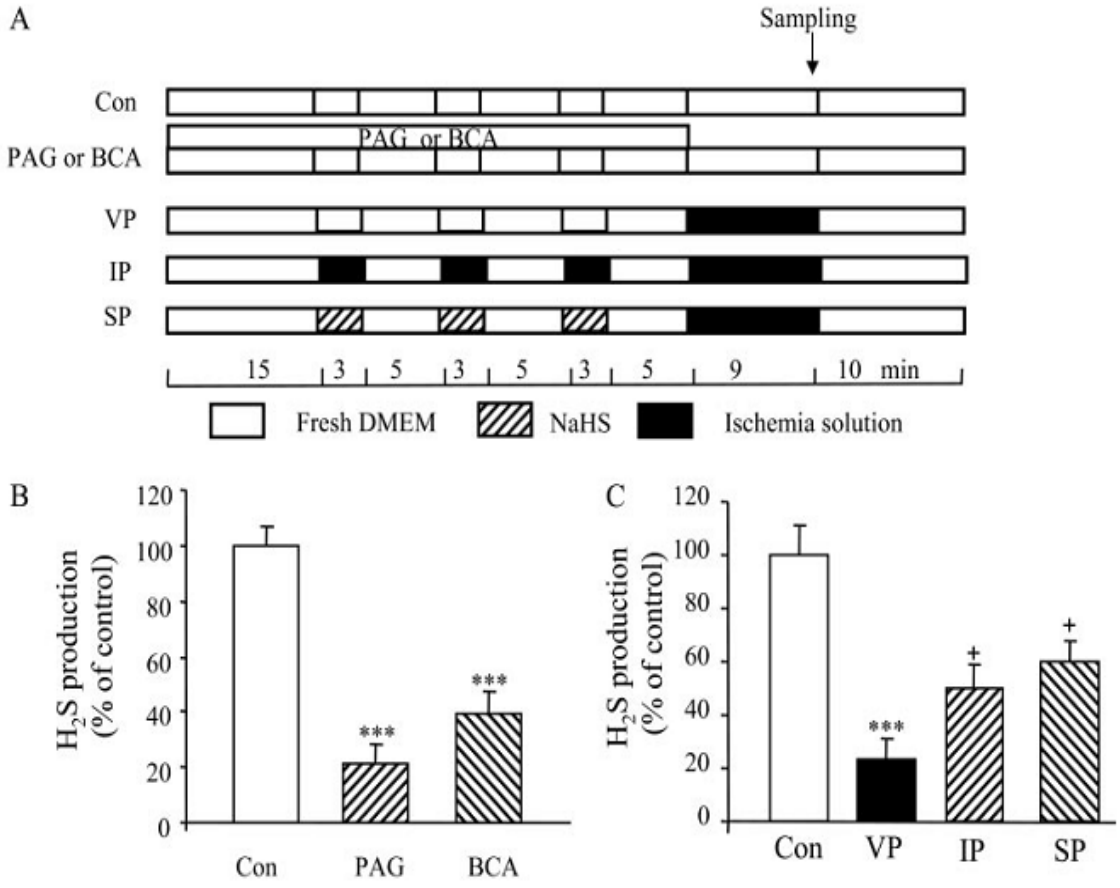


Figure 3-6. Effect of CSE inhibitors, ischemia, IP and SP on H_2S production in the rat ventricular myocytes. A, Experimental design. B, PAG and BCA decreased endogenous H_2S production. Mean±SEM; n=5. ***P<0.001 *vs* Control (Con). C, IP and SP attenuated the effect of ischemia on endogenous H_2S production. Mean±SEM; n=5-10. ***P<0.001 *vs* Control (Con), ^{+}P<0.05 *vs* VP.

3.3.7. Effects of H_2S synthesis inhibitors, IP and SP on H_2S levels in the culture medium of cardiac myocytes

I first observed whether PAG and BCA treatment for 40 min inhibited endogenous H_2S production in these experiments. As shown in Figure 3-6B, both PAG and BCA significantly decreased H_2S production by 78.8±7.1 % (n=5) and 60.4±7.6% (n=5), respectively.

To further investigate the hypothesis that endogenous H_2S may mediate the cardioprotection associated with IP, H_2S concentration in cell culture medium after 9 minutes of ischemia was determined. The experimental procedures are shown in Figure 3-6A and described in Methods. As shown in Figure 3-6B, ischemia for 9 min (VP group) significantly decreased (23.7±6.9 % (n=10, $p<0.001$) H_2S level in the VP group, suggesting that endogenous H_2S production is markedly decreased during ischemia. Interestingly, preconditioning with three cycles of ischemia or NaHS (100 μM) significantly attenuated the inhibitory effect of ischemia on H_2S production (IP: 49.6±9.5%; SP: 59.5±8.6%; n=5, $p<0.05$ vs ischemia group). These data suggest that both IP and SP may be able to reverse the inhibitory effect of ischemia on H_2S production.

3.4. Discussion

In the present study, I have observed a cardioprotective effect of exogenous application of NaHS. I observed that preconditioning with 100 μM NaHS attenuated arrhythmias in the isolated Langendorff-perfused heart (subjected to low-flow ischemia insults), increased cell viability and improved cell function in cardiac myocytes during ischemia/reperfusion. Our data clearly suggest that, at physiological concentrations, H_2S produces a cardioprotective effect.

Subsequently, I examined the potential role of endogenous H_2S in cardioprotection due to IP. Treatment of cardiac myocytes with either PAG or BCA markedly decreased endogenous H_2S production and significantly attenuated the protective effect of IP in the isolated rat heart and cardiac myocytes. Moreover, I also observed that H_2S production was decreased when ventricular myocytes were subjected to ischemia. Both IP and SP significantly attenuated the inhibitory effect of ischemia on H_2S production. Taken together, our data provide the first evidence that endogenous H_2S plays an important role in protecting heart function.

Ischemia/reperfusion-induced arrhythmias originate from a series of complex cellular and humoral reactions. The primary causes are considered to be the endogenous metabolites produced and accumulated in the myocardium during reperfusion. These various metabolites include, for example, reactive oxygen species (ROS), calcium, thrombin and platelet activating factor. H_2S may protect the heart against arrhythmias by scavenging ROS (Geng et al., 2004a) and opening K_{ATP} channel (Zhao et al., 2001) which reduce calcium influx and shortens action potential duration (APD). During

ischemia, H_2S production was markedly decreased. So this effect may increase harmful chemical substances such as ROS, which may, in turn, modulate cellular electrophysiology causing the complex changes at the level of ion channels and induce the arrhythmias. After preconditioning, both IP and SP could stimulate the heart to produce more endogenous H_2S and therefore protect the hearts. Blockade of endogenous H_2S synthesis increased both the duration of ischemia/reperfusion-induced arrhythmias and the severity of the arrhythmias. Thus, these data suggest that the endogenous H_2S system may mediate the cardioprotection induced by ischemic preconditioning.

In the present study, I also investigated the signaling mechanism underlying the cardioprotection of SP and IP. Both chelerythrine and BSM, (two specific PKC inhibitors) attenuated the protective effect of SP and IP, thereby suggesting that PKC may mediate the cardioprotection caused by both SP and IP. This is consistent with a previous observation that PKC plays an important role in mediating IP (Gross and Peart, 2003). During IP, PKC stimulation is secondary to activation of Gq or Gi/o protein coupled receptor (Eisen et al., 2004). The mechanism(s) by which PKC is activated during SP is unclear. However, an effect to open of K_{ATP} channels may be involved. This is supported by previous findings which showed that H_2S opens K_{ATP} channels in vascular smooth muscle cells (Zhao et al., 2001). Indeed, activation of PKC and K_{ATP} channels may be co-dependent (Baxter et al., 1995; Gross and Peart, 2003). Since protection provided by direct K_{ATP} channel openers may be abolished by PKC antagonists and vice versa, which implies that activation of PKC and K_{ATP} channels are both codependent and necessary for cardioprotection (Gaudette et al., 2000). Additional

experiments are needed to determine whether opening of K_{ATP} channels is an event upstream of PKC activation.

K_{ATP} channels are well known to play an important role in the cardioprotection induced by IP. However, the subtype of K_{ATP} channel which confers cardioprotective activity is still controversial. Since the first evidence of a role of the K_{ATP} channels in acute IP is presented (Gross and Auchampach, 1992) in the canine heart, results obtained in a number of studies using a variety of different models and species supported that the possibility sarcK_{ATP} channels triggered or mediated the cardioprotective effects of IP. Thus, IP and K_{ATP} channel openers shorten action potential duration (APD) (Noma, 1983; Tan et al., 1993), whereas K_{ATP} channel blockers attenuate the effect of IP on APD shortening (Cole et al., 1991; Yao and Gross, 1994). More evidence for the involvement of sarcK_{ATP} channel was provided by Suzuki and colleagues (Suzuki et al., 2002). They demonstrated that cardioprotection due to IP was blocked by HMR-1098 (a putative sarcK_{ATP} channel blocker) but not by 5-HD. However, Sasaki et al found MCC-134, a novel pharmacological agent which opens sarcK_{ATP} channels and blocks mitoK_{ATP} channels, attenuated the effects of IP. These data suggest that the sarcK_{ATP} channel may not be totally accountable for the protective effects afforded by IP. In the present study, I found that the subtypes of K_{ATP} channels involved in the cardioprotection of IP and SP may differ. I observed that various K_{ATP} channel blockers, including glibenclamide, 5-HD and HMR-1098, attenuated the cardioprotection of IP, which suggests that both sarcK_{ATP} and mitoK_{ATP} are involved. However, selective blockade of mitoK_{ATP} channels with 5-HD had no effect on the protective effect of SP on cell viability and cell function, whereas glibenclamide and

HMR-1098 significantly attenuated this effect. Thus, these data suggested that sarcK_{ATP}, but not mitoK_{ATP}, may mediate the cardioprotection of SP.

Opening of the sarcK_{ATP} channel induced by SP would be expected to enhance shortening of the cardiac APD by accelerating phase 3 repolarization. Thereby inhibiting Ca^{2+} entry into the cell via L-type channels and preventing Ca^{2+} overload. Furthermore, the slowing of depolarization would also be expected to reduce Ca^{2+} entry and slow or prevent the reversal of the Na^{+}-Ca^{2+} exchanger. All of these actions may increase cell viability via a reduction in Ca^{2+} overload during ischemia and early reperfusion.

Both PAG and BCA have been widely used to inhibit CSE activity and endogenous H_2S production. PAG causes an irreversible inhibition of CSE activity --- in vitro (Johnston et al., 1979) and in vivo (Mok et al., 2004; Porter et al., 1996; Uren et al., 1978), whereas BCA is a reversible inhibitor of CSE (Pfeffer and Ressler, 1967; Uren et al., 1978). Despite the widespread use of both PAG and BCA to inhibit H_2S formation there is a possibility that one or both of these compounds may produce effects by mechanism(s) which are unrelated to inhibition of CSE. However, this possibility seems unlikely since neither PAG nor BCA alone significantly affected cell viability or heart rhythm. Furthermore, co-administration of NaHS reversed the effect of both CSE inhibitors that both drugs attenuated the cardioprotection of IP on cardiac rhythm, implying that the effects of PAG and BCA most likely result from a decrease in endogenous H_2S formation.

In summary, it was demonstrated, for the first time, that endogenous H_2S contributes to the cardioprotection conferred by IP and pharmacological

preconditioning with the H_2S donor, NaHS confers cardioprotection. SarcK_{ATP} channel and PKC may be involved in the cardioprotective effect of H_2S. In addition, mitoK_{ATP} may also be involved in the cardioprotective effect induced by IP.

Chapter 4 Role of hydrogen Sulfide in the Cardioprotection Induced by Ischemic Postconditioning

4.1. Introduction

In chapter 3, it was demonstrated that endogenous H_2S mediated the cardioprotection induced by ischemic preconditioning, and a H_2S donor was an effective pharmacological preconditioning agent. However, ischemic preconditioning has a major limitation in that brief ischemia maneuver or its mimetic, which can trigger pharmacological preconditioning, has to be applied before the index ischemia insult. This has lead to the introduction of the concept of ischemic postconditioning (IPostC) by Na's (Na et al., 1996) and Vinten-Johansen's groups (Zhao et al., 2003). IPostC is defined as the phenomenon where rapid intermittent interruptions of blood flow in the early phase of reperfusion resulted in a reduced myocardial injury (Zhao and Vinten-Johansen, 2006). This maneuver has produced promising protection against ischemia-reperfusion injury in mice (Kin et al., 2005), rats (Kin et al., 2004), rabbits (Yang et al., 2004b), dogs (Zhao et al., 2003) and in human patients (Staat et al., 2005).

Exogenous H_2S was shown to activate several pro-survival kinases such as ERK1/2 (Yang et al., 2004a; Yang et al., 2006; Zhi et al., 2007), Akt (Hu et al., 2007d) and PKC (Pan et al., 2007), which are the mediators of IPostC. Although the complete signaling mechanisms of H_2S remains unclear, the general understanding on the cardioprotective effect of H_2S up to date has led us to hypothesize that endogenous H_2S may contribute to the protective effect of IPostC. As such, the aim of our study was to examine whether endogenous H_2S plays a role in mediating the IPostC via activation of

pro-survival kinases. In addition, the role of exogenous H_2S to serve as a potential candidate to trigger pharmacological postconditioning was also studied.

4.2. Materials and methods

4.2.1. Measurement of cardiodynamic functions

Cardiodynamic parameters were measured with a pressure transducer connected to a PowerLab system (ADInstruments, Australia). An incision was made in the left atrium and a fluid-filled latex balloon connected to the pressure transducer was inserted and positioned in the left ventricular cavity for continuous assessment of cardiodynamic function. The balloon was initially inflated to an end-diastolic pressure of 5 – 10 mmHg and thereafter the balloon volume was held constant. The hearts were perfused at a constant flow rate of 12 ml/min. Cardiodynamic data were analyzed using a Data Acquisition System (PowerLab System, AD Instruments, Australia). Left ventricular end diastolic pressure (LVeDP) was represented by the minimum pressure recorded during diastoles, left ventricular developed pressure (LVDP) was calculated as the difference between left ventricular systolic pressure and left ventricular diastolic pressure, contractility (+dP/dt) was represented by the maximum gradient during systoles and compliance (-dP/dt) was represented by the minimum gradient during diastoles.

4.2.2. Measurement of myocardial infarction size

Regional ischemia was induced by ligating the left main coronary artery (LAD) for 40 minutes. The ligation was released during the 2 h reperfusion. After 2 h reperfusion, the infarct–risk volume ratio was determined. As described previously (Hausenloy et al.,

2005), the heart was stained by slowly infusing 1 ml of Evan's blue (3% w/v in phosphate-buffered saline) via the aorta, followed by perfusion with Krebs bicarbonate buffer to wash out unbound stains. The heart was then immediately removed from the perfusion apparatus, weighed, and stored overnight at −20°C. The frozen heart was thereafter cut into five or six transverse sections (approximately 2 mm in thickness) across the long axis, stained with 1% TTC in phosphate buffer (pH 7.4) for 20 min at 37°C, and then fixed in 10% formalin overnight. The size of the myocardial infarction (appearing as pale color) was quantified by ImageJ (1.32j, National Institutes of Health, USA) and calculated as percentage of area at risk which is the area not stained by Evan's blue.

4.2.3. Western blot analysis

After 1 hr reperfusion, the ventricular tissues of the heart which had undergone global ischemia were freeze-clamped in liquid nitrogen and stored at -80 ºC for further analysis. Phosphorylation state of Akt and eNOS, and translocation of PKCα, PKCδ and PKCε, was assessed with standard western immunoblotting. Briefly, 25 mg of tissues were minced and homogenized in ice-cold lysis buffer containing (mM) 25 Tris·HCl pH 7.5, 150 NaCl, 5 EDTA, 10 NaF, 1 Na3VO4, 1% NP-40 and 0.4% deoxycholic acid supplemented with protease inhibitor cocktail tablet (Roche Diagnostics, Penzberg, Germany). A cell fractionation technique was adopted from the literature (Weber et al., 2005). Homogenates were centrifuged at 1,000xg for 10 min. The cytosolic fraction of the proteins were obtained by collecting the supernatant and centrifuged at 16,000xg to maximize protein extraction. The membrane fraction was obtained by treating the pellet with lysis buffer supplemented with 1% Triton-X followed by centrifugation at

16,000xg. Protein concentrations were determined using the modified Lowry method. Proteins were denatured with SDS-sample buffer and epitopes were exposed by boiling the protein samples at 100 oC for 5 min. Equal amount of proteins were loaded and separated by electrophoresis on 12% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. The membrane was first probed with the primary antibody that recognizes phospho-Akt (p-Akt, serine 473), total Akt, phospho-endothelial NO synthase (p-eNOS, serine 1117), total eNOS, PKCα, PKCδ or PKCε and then with a horseradish peroxidase-conjugated goat-anti-rabbit IgG secondary antibody. Immunoreactivity was detected using the enhanced chemiluminescence method. Protein levels were normalized to the VPostC group.

4.2.4. Measurement of H_2S-synthesis enzymes activity

Tissue H_2S production rate was measured essentially as described previously (Webb et al., 2008; Yong et al., 2008b). Briefly, a snap-frozen 100mg of rat ventricular tissues from VPostC, IPostC and IPostC+PAG groups were homogenized in 1 ml of 100 mM potassium phosphate buffer, pH 7.4. The assay mixtures (500 µl) contained 460 µl of tissue homogenate, 10 mM L-cysteine, and 2 mM pyridoxal 5'-phosphate. Incubation was carried out in tightly sealed Eppendorf vials. After incubation (37ºC, 30 min), zinc acetate (1% w/v, 250 µl) was added to trap the generated H_2S followed by trichloroacetic acid (10% w/v, 250 µl) to stop the reaction. Next, N,N-dimethyl-p-phenylenediamine sulfate (20 mM, 133 µl) and FeCl3 (30 mM; 133 µl) was added and centrifuged under 14,000 g. After 10 min, absorbance at 670 nM of 200 µl aliquots of the supernatant was determined. The H_2S concentration of samples was calculated using a calibration curve of NaHS (3.125 – 200 µM), and the results were expressed as

nanomoles of H_2S produced per milligram of soluble protein per 30 min. Protein was determined with NanoDrop Spectrophotometer (NanoDrop technology, ND-1000, DE, USA).

4.2.5. Experimental Protocol

Isolated rat hearts were perfused and stabilized for at least 20 min before data recording. Global ischemia was mimicked by stopping the perfusion of Krebs bicarbonate buffer, i.e. perfusion rate = 0 ml/min. The hearts were randomly divided into nine groups according to the perfusion protocol as shown in Fig. 1a, 2a, 5a, 9a and 10a. Vehicle postconditioning group (VPostC) and ischemic postconditioning group (IPostC) serve as the negative and positive controls in this study. In VPostC and IPostC groups, hearts experienced 20 minutes equilibration followed by 40 min global ischemia. Upon reperfusion, IPostC hearts were treated with 6 cycles of 10 s reperfusion and 10 s no flow ischemia (total intervention time of 2 min) whereas VPostC hearts did not receive any additional treatment. In IPostC+PAG group, hearts were pre-treated with 2 mM PAG, a CSE inhibitor, 15 min before, during and 2 min after ischemia treatment (Fig. 1a & 2a). Two protocols of H_2S postconditioning were adopted: SPostC group hearts were treated with 6 cycles of 10 s reperfusion and 10 s 100 μM NaHS, a H_2S donor, intermittently whereas SPostC2 group hearts were treated with 100 μM NaHS for two minutes continuously (Figure 4-5). NaHS treatments were performed by directly injecting 100 μM NaHS into perfusing Krebs bicarbonate buffer to avoid flow interruption caused by switching of stopcocks. In four parallel groups (SPostC-LY, SPostC2-LY, SPostC-CHE and SPostC2-CHE), hearts were treated with 15 μM

LY294002 (PI3K/Akt inhibitor), or 10 μM chelerythrine (PKCs inhibitor) 10 min before, during and 5 min after ischemia insults (Fig. 9a & 10a).

4.2.6. Other methods

Isolated prefused rat heart preparation and H_2S concentration and its generating enzyme activity have been described in the Materials and Methods in Chapter 2.

4.2.7. Statistical analysis

All values were expressed as mean ± SEM. Cardiodynamic data were assessed with one-way ANOVA followed by Tukey's post hoc test. Immunoreactivity of each protein of interest was normalized to VPostC and compared among the groups by ANOVA and subsequent Newman-Keuls post hoc test. Differences were considered statistically significant when $p<0.05$.

4.2.8. Drugs and chemicals

NaHS and D-L-propargylglycine (PAG) were purchased from Sigma Chemical Co, USA. Chelerythrine chloride and LY294002 were purchased from Calbiochem, Darmstadt, Germany. Polyclonal anti-phospho-Akt rabbit IgG and polyclonal anti-total-Akt rabbit IgG were purchased from Cell Signaling Technology Inc, USA. Polyclonal anti-PKCε rabbit IgG antibody was purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Chelerythrine chloride and LY294002 was dissolved in dimethyl sulphoxide (DMSO) and added to the Krebs bicarbonate buffer such that the final DMSO concentration was less than 0.1%. All other chemicals were dissolved in deionized water.

4.3. Results

4.3.1. Activity of H_2S-synthesis enzymes in ischemia/reperfusion with and without IPostC treatment

The experimental protocols are shown in Figure 4-1A. H_2S-generating enzyme activities in the heart tissues were measured at the end of 6 cycles of postconditioning treatment in IPostC group or after 2 min of reperfusion in VPostC group. As shown in Figure 4-1B, H_2S-synthesis enzyme activity was significantly decreased in VPostC group, suggesting that ischemia may inhibit endogenous H_2S production. This is consistent with our previous findings that ischemia decreases H_2S production in isolated cardiomyocytes (Bian et al., 2006; Pan et al., 2006). However, the decreased enzyme activity was dramatically reversed and enhanced by IPostC, suggesting that IPostC treatment can strongly stimulates the production of endogenous H_2S, which may serve as a mediator for the cardioprotective effects of IPostC.

It was also found that the effect of IPostC on H_2S-generating enzyme activity was blocked by PAG (Figure 4-1B), confirming that PAG can serve as an inhibitor of CSE in our experimental model. To test whether PAG can be safely used in the functional studies, I also examined its effect on cardiodynamic function in the isolated rat hearts. As shown in Figure 4-1C & 4-1D, administration of PAG (2 mM) for 40 min did not impair LVDP. PAG was therefore employed in the following experiments to test the involvement of H_2S in the cardioprotection of IPostC.

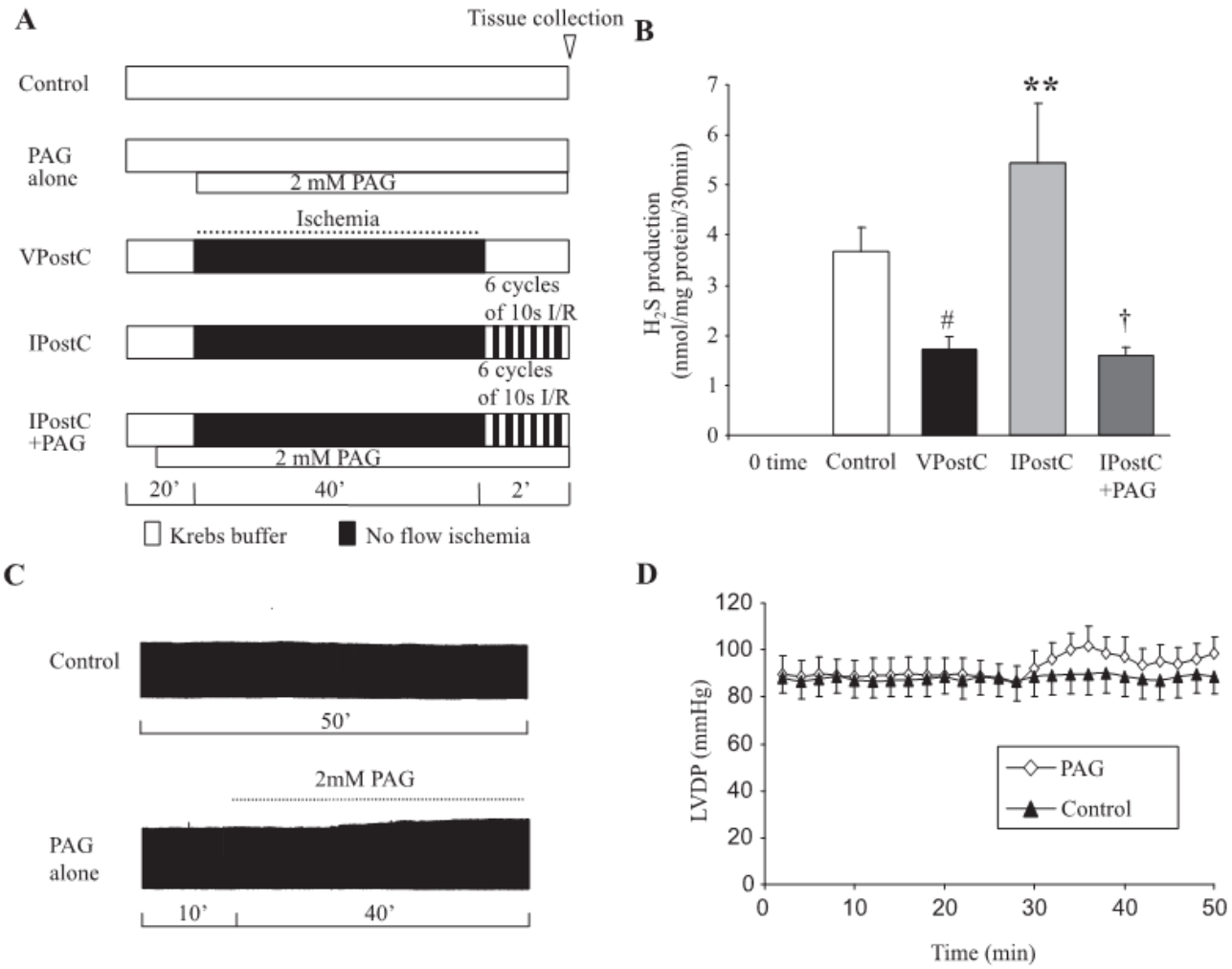


Figure 4-1 Activity of H_2S-generating enzymes with and without IPostC and the effect of PAG on cardiodynamic function. (a) Experimental protocols. Open field: Krebs solution; solid field: no flow ischemia. PAG, a H_2S synthesis inhibitor, was given 15 min before, during and 2 min after ischemia treatment. (b) Effect of IPostC on the activity of H_2S-synthesis enzymes with and without IPostC. Mean data showing the H_2S production produced by the H_2S-generating enzyme in the homogenates of rat ventricular tissue in the presence of substrate and co-factors. 0 time group serves a negative control, representing the start of the experiment when no H_2S is produced. The next four bars show the production of H_2S after 30 min. All values are mean ± S.E.M. (n=8), $^{\#}p<0.05$ VPostC *vs*. control, **$p<0.01$ VPostC *vs*. IPostC, $^{\dagger}p<0.05$ IPostC *vs*. IPostC+PAG. (c-d) Effect of PAG on cardiodynamic function. (c) Representative tracings showing the mechanical performance of isolated heart during perfusion of 2mM PAG for 40 min (lower panel) as compared to Kreb's solution only (upper panel). (d) Mean data of LVDP showing that 2 mM PAG alone did not alter the LVDP significantly.

4.3.2. Role of endogenous H_2S in the cardioprotection induced by IPostC

Cardiodynamic function was measured to test the involvement of endogenous H_2S in the cardioprotection induced by IPostC. The experimental protocols are shown in Figure 4-2A and described in the Materials and Methods. As shown in Fig. 4-2B & 4-2C, IPostC significantly protected the heart by resuming the functional performance after ischemia as compared to VPostC when assessed with LVeDP, LVDP and ±dP/dt. Inhibition of endogenous H_2S synthesis with 2 mM PAG from 15 minutes before up to 2 minutes after ischemia significantly diminished the cardioprotective effect of IPostC by elevating LVeDP and reducing both LVDP and +dP/dt. However, no significant change in –dP/dt was observed, implying a minor role played by endogenous H_2S in heart relaxation.

4.3.3. Role of endogenous H_2S in the activation of PKC isoforms triggered by IPostC

PKC is an important trigger/mediator in the cardioprotection induced by IPostC (Philipp et al., 2006; Zatta et al., 2006). The PKC family consists of at least 10 isoforms, among which PKC-α, ε and δ are the prominent isoforms expressed in the heart (Mackay and Mochly-Rosen, 2001b). I therefore examined whether endogenous H_2S mediates the activation of these three PKC isoforms during IPostC. As shown in Fig. 4-3, translocation of different PKC isoforms (α, δ and ε) from cytosol to membrane was observed in IPostC treatment group, indicating IPostC stimulates these PKC isoforms. Inhibition of endogenous H_2S synthesis with PAG significantly reversed the translocation of PKCα and PKCε but not PKCδ (Fig. 4-3). These data suggest that endogenous H_2S played a part in IPostC-triggered activation of PKCε and PKCα.

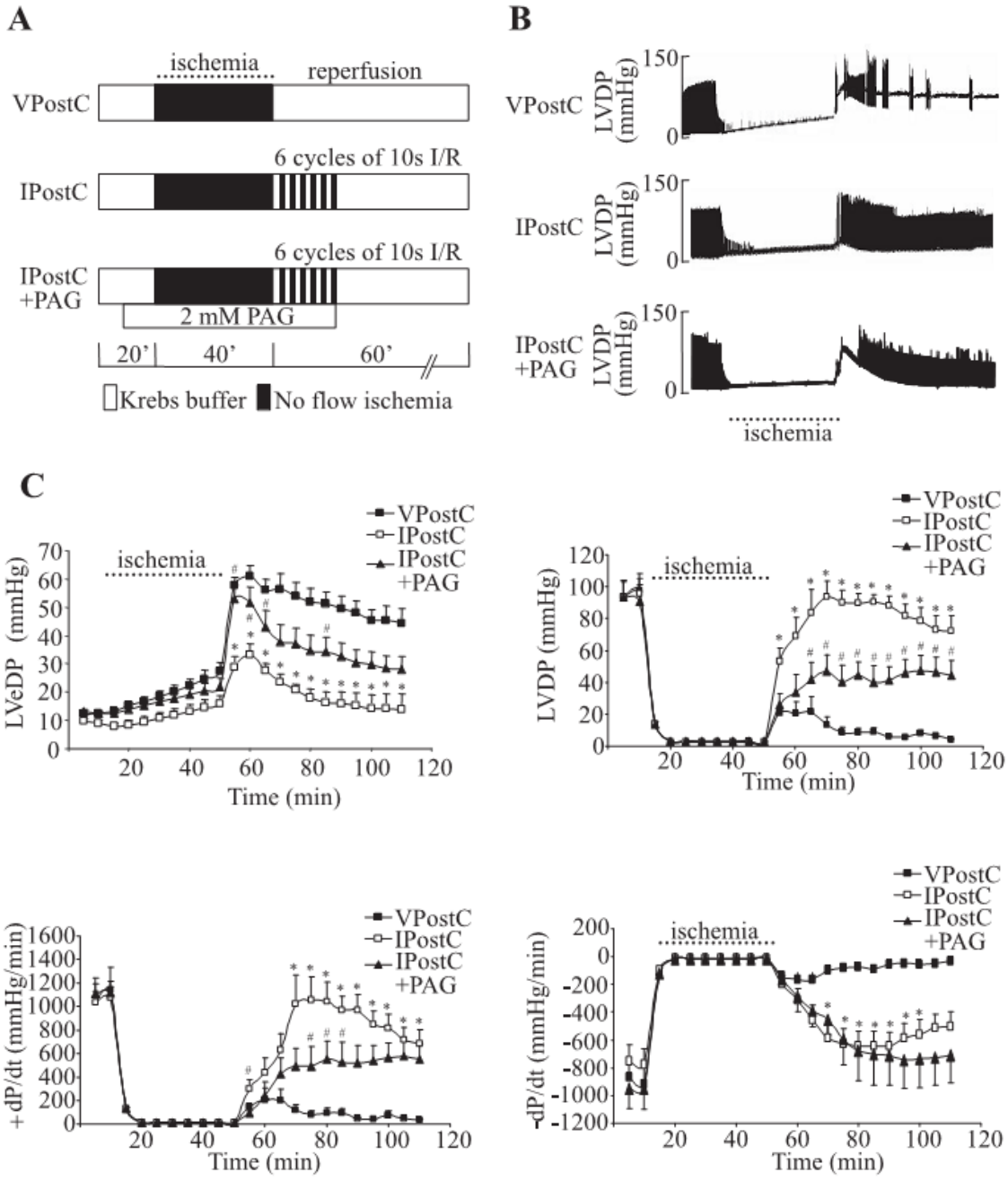


Figure 4-2 Effect of IPostC on cardiodynamics in the presence and absence of PAG, a H_2S synthesis inhibitor. (a) Experimental protocols. Open field: Krebs solution; solid field: no flow ischemia. PAG was given 15 min before, during and 2 min after ischemia treatment. (b) Representative tracings showing the mechanical performance of isolated rat hearts during 40 minutes ischemia and subsequent one hour reperfusion in VPostC, IPostC and IPostC+PAG groups. (c) Mean data of left ventricular end-diastolic pressure (LVeDP) (upper left panel), left ventricular developed pressure (LVDP) (upper right panel), contractility (+dP/dt) (lower left panel) and compliance (-dP/dt) (lower right panel). All values are mean ± S.E.M. (n=6), *p<0.05 VPostC *vs*. IPostC, $^{\#}$p<0.05 IPostC *vs*. IPostC+PAG.

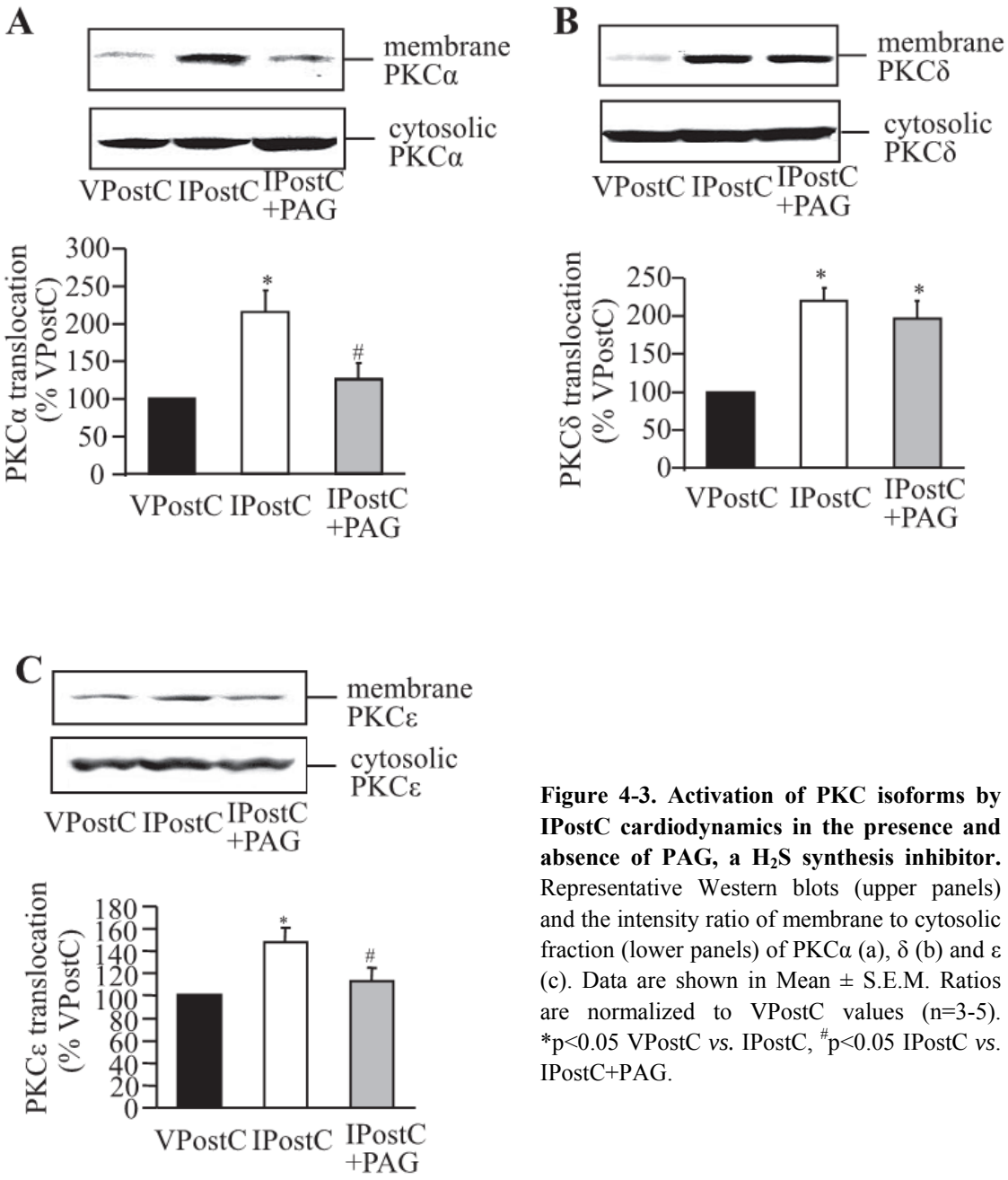


Figure 4-3. Activation of PKC isoforms by IPostC cardiodynamics in the presence and absence of PAG, a H_2S synthesis inhibitor. Representative Western blots (upper panels) and the intensity ratio of membrane to cytosolic fraction (lower panels) of PKCα (a), δ (b) and ε (c). Data are shown in Mean ± S.E.M. Ratios are normalized to VPostC values (n=3-5). *p<0.05 VPostC *vs.* IPostC, #p<0.05 IPostC *vs.* IPostC+PAG.

4.3.4. Role of endogenous H_2S in the activation of Akt and eNOS triggered by IPostC

Since IPostC was shown to trigger cardioprotection partly via activation of pro-survival kinase Akt (Tsang et al., 2004; Zhu et al., 2006) and eNOS (Tsang et al., 2004), I further examined whether inhibition of endogenous H_2S production could reverse phosphorylation of Akt and eNOS upon IPostC treatment. As shown in Fig. 4-4, IPostC significantly induced phosphorylation of Akt (Fig. 4-4A) and eNOS (Fig. 4-4B). This is consistent with the previous findings from other groups (Tsang et al., 2004; Zhu et al., 2006). However, inhibition of endogenous H_2S synthesis with PAG failed to attenuate IPostC-induced phosphorylation of Akt (Fig. 4-4A) and eNOS (Fig. 4-4B), suggesting that activation of both enzymes by IPostC was not solely via endogenous H_2S.

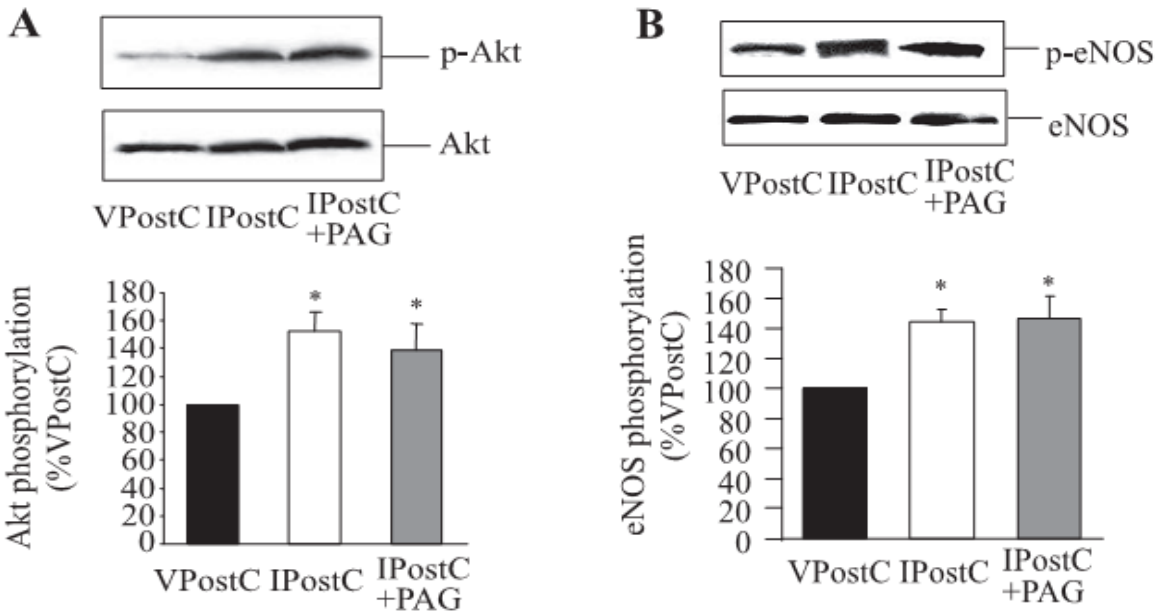


Figure 4-4. Activation of Akt and eNOS by IPostC in the presence and absence of PAG, a H_2S synthesis inhibitor. Representative Western blots (upper panels) and the relative intensity of phospho-Akt/total-Akt (a) & phosphor-eNOS/total eNOS (b) showing PAG failed to abolish the IPostC induced activation of Akt and eNOS. Data are shown in Mean ± S.E.M. Ratios are normalized to VPostC values (n=3-5), *p<0.05 VPostC *vs.* IPostC or IPostC+PAG.

4.3.5. H_2S postconditioning improves the cardiodynamic performance of isolated perfused rat heart after ischemia

This series of experiments were designed to determine whether postconditioning with exogenous H_2S was also able to produce cardioprotection. NaHS-reperfusion was used to substitute the six episodes of ischemia-reperfusion in IPostC. As shown in Fig. 4-5A, two pharmacological postconditioning protocols which were commonly adopted by other groups (Lu et al., 2006; Tissier et al., 2007) were used in the present study. Since NaHS at 100 μM exhibited the maximal cardioprotective effect from ischemia-induced injury in both cardiac myocytes (Pan et al., 2006) and isolated rat hearts (Hu et al., 2007d), 100 μM was therefore chosen to oberve the cardioprotection of NaHS postconditioning in the following experiments. In SPostC group, hearts received 6 cycles of 10 s reperfusion and 10 s NaHS infusion after ischemia, whereas in SPostC2 NaHS (100 μM) was given for two minutes continuously after ischemia. Fig. 4-5B and 4-5C showed that both SPostC and SPostC2 treatment significantly improved the cardiodynamics including LVeDP, LVDP and ±dP/dt during reperfusion after ischemia. The cardioprotective effect was comparable to that of IPostC.

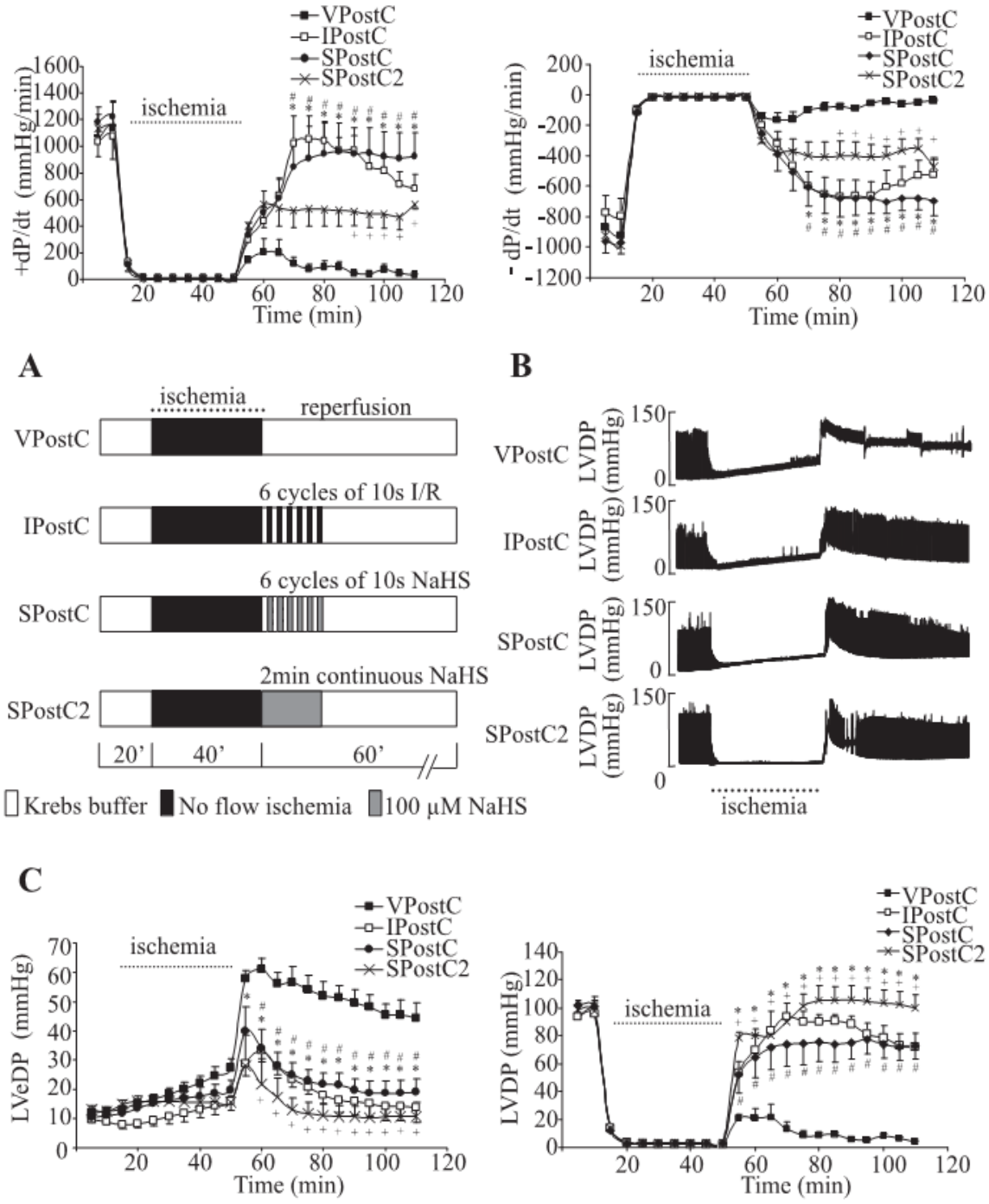


Figure 4-5. Effect of H_2S postconditioning on cardiodynamics. (a) Experimental protocols. Open field: Krebs solution; solid field: no flow ischemia; slashed field: Krebs solution with 100 μM NaHS. (b) Representative tracings showing the mechanical performance of isolated rat hearts during 40 minutes ischemia and subsequent 1 hour reperfusion in different groups. (c) Mean data of left ventricular end-diastolic pressure (LVeDP) (upper left panel), left ventricular developed pressure (LVDP) (upper right panel), contractility (+dP/dt) (lower left panel) and compliance (-dP/dt) (lower right panel). All values are mean ± S.E.M. (n=6), *p<0.05 VPostC *vs.* IPostC, $^{\#}$p<0.05 VPostC *vs.* SPostC, $^{+}$p<0.05 VPostC *vs.* SPostC2.

4.3.6. H_2S postconditioning limits myocardial infarct size of isolated perfused rat heart

To further confirm the cardioprotective effects of SPostC, infarct size was assessed in VPostC, SPostC and SPostC2 groups. Hearts received SPostC and SPostC2 treatment displayed 71% & 72% reductions, respectively, in infarct size per area-at-risk (SPostC: 12.7 ± 5%; SPostC2: 12.4 ± 3%) as compared with that in vehicle group (44.1 ± 5%, Fig. 4-6). These data confirm that H_2S may serve as a possible trigger for pharmacological postconditioning.

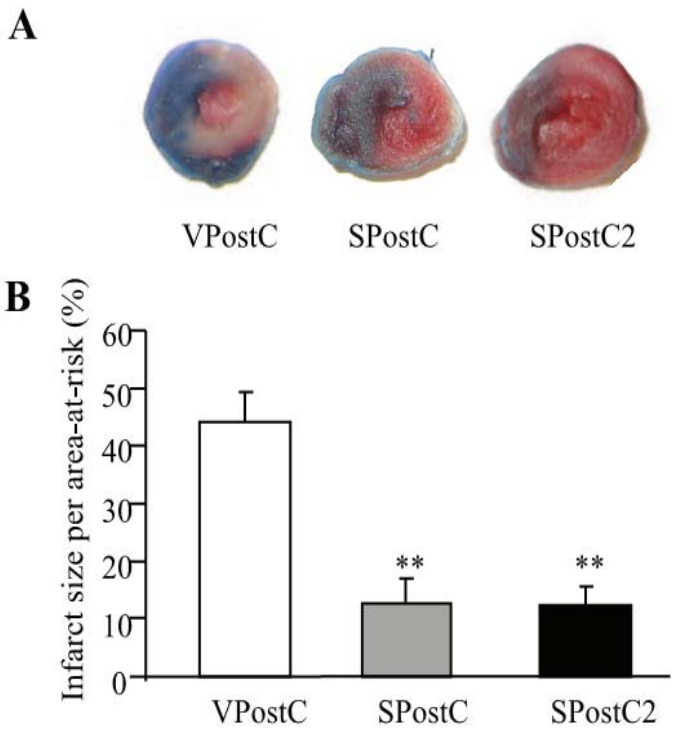


Figure 4-6. Effect of H_2S postconditioning on myocardial infarction. (a) Representative mid-myocardial cross sections of TTC-stained hearts in vehicle, SPostC and SPostC2 groups. Dark blue area indicates non-ischemic area. Total area of white (infracted tissue) and red (viable myocardium) represents area-at-risk. (b) Mean data showing that SPostC and SPostC2 significantly reduced the myocardial infarct size as compared to VPostC group. All values are mean ± S.E.M. (n=6-7), **p<0.01 VPostC *vs.*

4.3.7. H_2S postconditioning activates Akt, eNOS and PKC

The signaling mechanisms underlying the cardioprotection of H_2S postconditioning were also examined. I first determined the involvement of PKC isoforms (α, δ and ε) in the cardioprotection triggered by H_2S postconditioning. SPostC translocated all three isoforms of PKC, however, SPosC2 only induced PKCδ activation (Fig. 4-7), implying that SPostC and SPostC2 may induce cardioprotection via different signaling mechanisms.

Since both Akt and eNOS mediate the cardioprotection of IPostC, phosphorylation of Akt and eNOS was also examined. When compared to VPostC, both SPostC and SPostC2 treatment induced significant phosphorylation of Akt (Fig. 4-8A). Interestingly, SPostC2, but not SPostC, also induced the phosphorylation of eNOS (Fig. 4-8B).

4.3.8. Roles of Akt and PKC in the cardioprotection triggered by H_2S postconditioning

To further confirm the role of Akt and PKC in the cardioprotection induced by H_2S postconditioning, specific inhibitors of PI-3-Kinase (PI3K)/Akt and PKC were administered in both SPostC and SPostC2 groups. As shown in Fig. 4-9 & 4-10, inhibition of either PI3K/Akt pathway with LY294002 (15 μM) or PKC with chelerythrine (10 μM) during the H_2S infusion period significantly reversed the protective effect on contractile function in both SPostC and SPostC2 groups. These data suggest that activation of both PI3K/Akt and PKC pathways are essential in the cardioprotection induced by both SPostC and SPostC2.

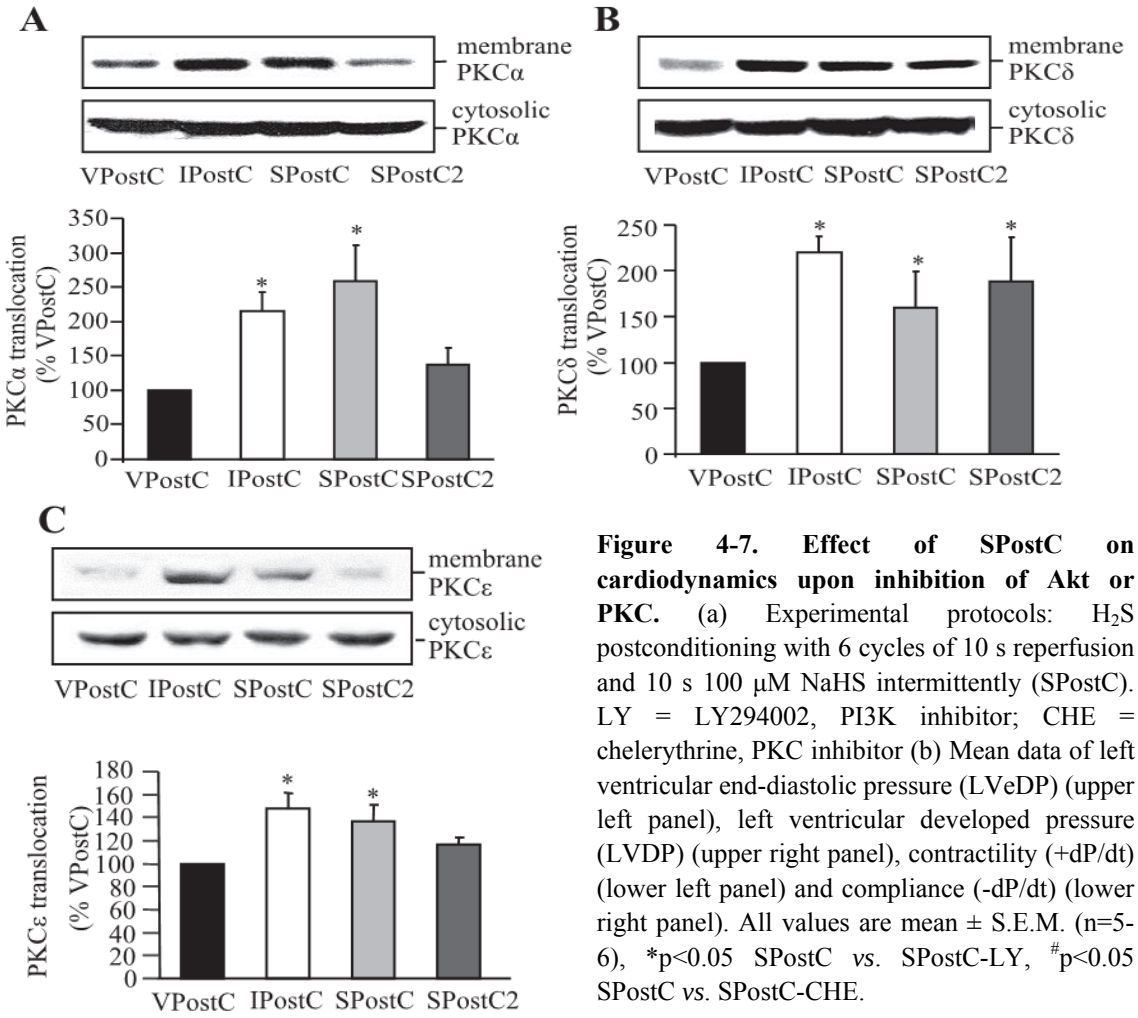


Figure 4-7. Effect of SPostC on cardiodynamics upon inhibition of Akt or PKC. (a) Experimental protocols: H_2S postconditioning with 6 cycles of 10 s reperfusion and 10 s 100 μM NaHS intermittently (SPostC). LY = LY294002, PI3K inhibitor; CHE = chelerythrine, PKC inhibitor (b) Mean data of left ventricular end-diastolic pressure (LVeDP) (upper left panel), left ventricular developed pressure (LVDP) (upper right panel), contractility (+dP/dt) (lower left panel) and compliance (-dP/dt) (lower right panel). All values are mean ± S.E.M. (n=5-6), *p<0.05 SPostC *vs*. SPostC-LY, #p<0.05 SPostC *vs*. SPostC-CHE.

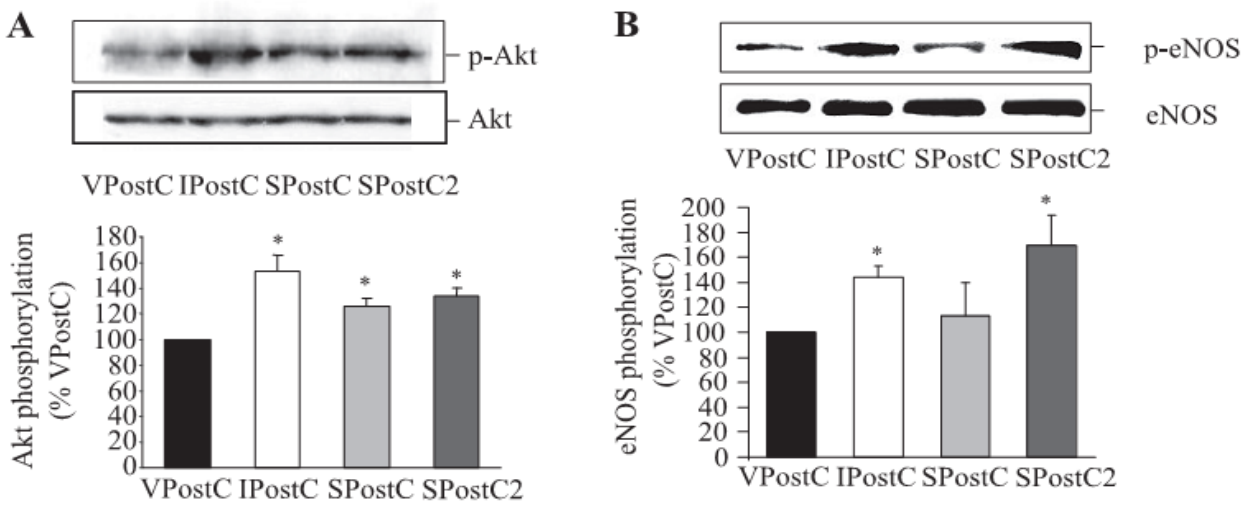


Figure 4-8. Activation of Akt and eNOS induced by IPostC, SPostC and SPostC2. Representative Western blots (upper panels) and the relative intensity of phospho-Akt/total-Akt (a) & phospho-eNOS/total eNOS (b) showing SPostC2 stimulated both Akt and eNOS, whereas SPostC only activated Akt. Data are shown in Mean ± S.E.M. Ratios are normalized to VPostC values, (n=3-5). *p<0.05 *vs.* VPostC.

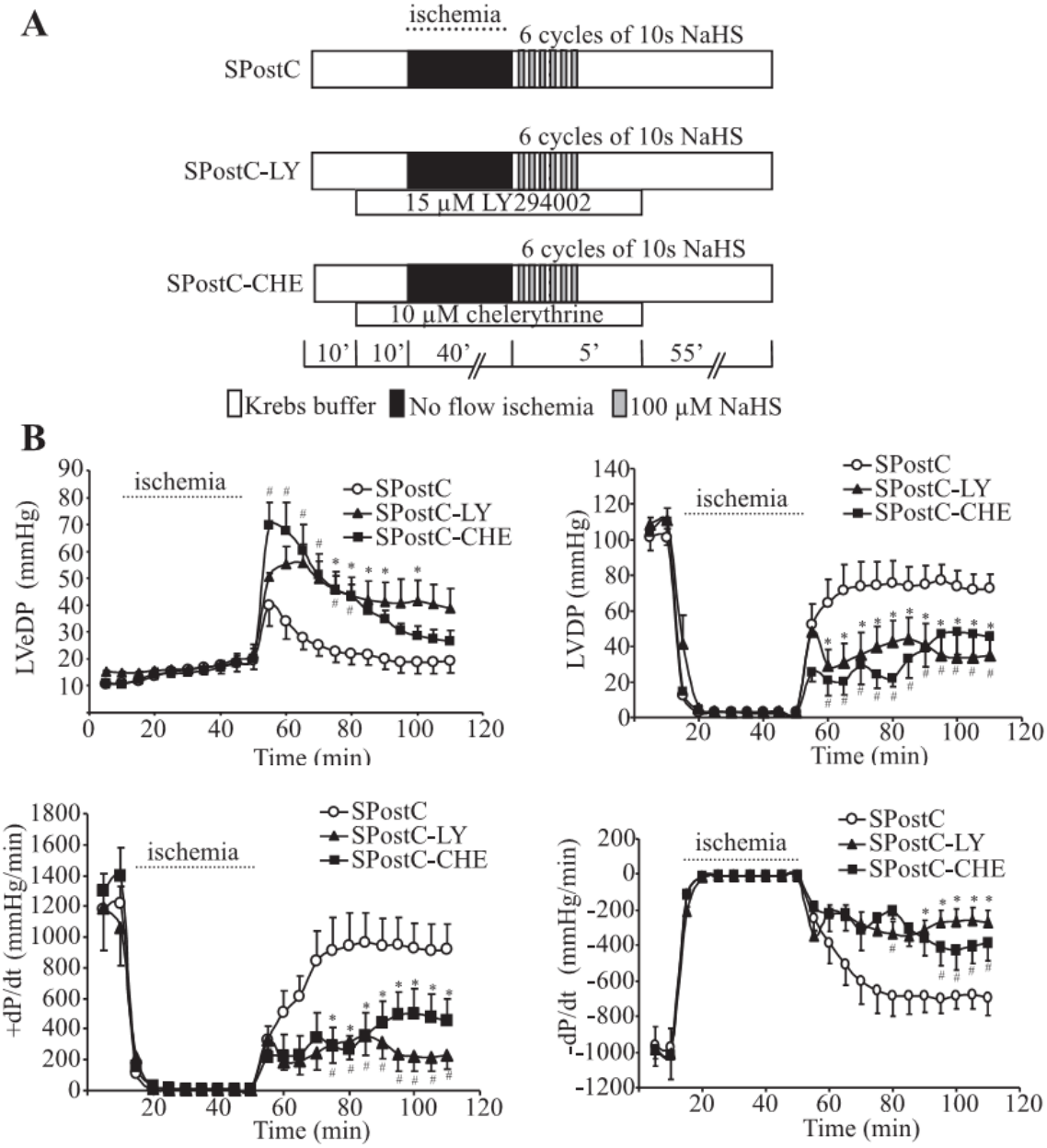


Figure 4-9. Effect of SPostC on cardiodynamics upon inhibition of Akt or PKC. (A) Experimental protocols: SPostC. LY = LY294002, a PI3K inhibitor; CHE = chelerythrine, a PKC inhibitor (B) Mean data of left ventricular end-diastolic pressure (LVeDP) (top left), left ventricular developed pressure (LVDP) (top right), contractility (+dP/dt) (bottom left) and compliance (-dP/dt) (bottom right). All values are mean ± S.E.M. (n=5-6), *p<0.05 SPostC *vs*. SPostC-LY, $^{\#}$p<0.05 SPostC *vs*. SPostC-CHE.

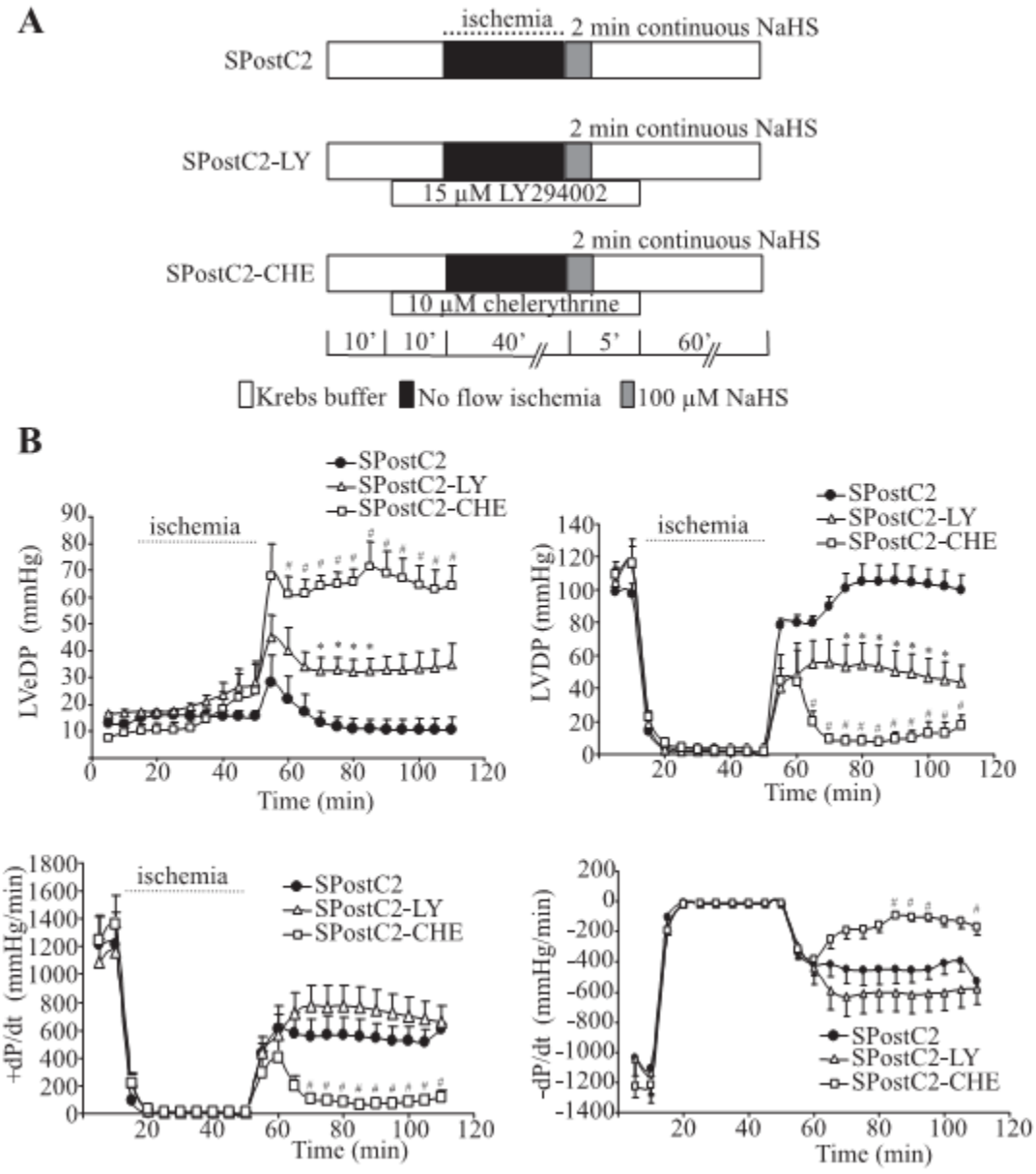


Figure 4-10. Effect of SPostC2 on cardiodynamics upon inhibition of Akt or PKC. (a) Experimental protocols: H_2S postconditioning with 100μM NaHS for two minutes continuously (SPostC2). LY = LY294002, a PI3K inhibitor; CHE = chelerythrine, a PKC inhibitor (b) Mean data of left ventricular end-diastolic pressure (LVeDP) (upper left panel), left ventricular developed pressure (LVDP) (upper right panel), contractility (+dP/dt) (lower left panel) and compliance (-dP/dt) (lower right panel). All values are mean ± S.E.M. (n=5-6), *$p<0.05$ SPostC2 *vs*. SPostC2-LY, $^{\#}p<0.05$ SPostC2 *vs*. SPostC2-CHE.

4.4. Discussion

The main objective of this study was to unveil the role of endogenous H_2S in the cardioprotection of IPostC and to explore the feasibility of postconditioning with exogenous H_2S to trigger cardioprotection. Cardiodynamics of isolated perfused rat heart and myocardial infarction were chosen as the parameter to assess the functional performance of the heart after ischemia. In the present study, I found that IPostC significantly improved the heart contractile function after ischemia. This is consistent with the previous findings that IPostC protects the heart from lethal ischemia reperfusion injury when assessed by infarct size (Darling et al., 2005; Kin et al., 2004; Yang et al., 2005b), cardiodynamic performance (Crisostomo et al., 2006; Fantinelli and Mosca, 2007; Kin et al., 2005; Zhu et al., 2006), cellular injury index (Sun et al., 2005; Wang et al., 2006) etc. Inhibition of endogenous H_2S synthesis with PAG, an irreversible inhibitor of CSE, partly attenuated the cardioprotective effect of IPostC. More importantly, I found that IPostC treatment stimulated the activity of H_2S-generating enzymes in the early phase of reperfusion. These observations suggest a role for endogenous H_2S in the phenomenon.

Interestingly, inhibition of endogenous H_2S synthesis with PAG did not block all signaling pathways stimulated by IPostC. PAG attenuated IPostC-induced translocation of PKCε and PKCα, but failed to affect the phosphorylation of Akt, eNOS and translocation of PKCδ. These data suggest a central dominant role for endogenous H_2S in the activation of the pro-survival PKCε and PKCα, whereas activation of Akt, eNOS and PKCδ by IPostC might not be solely dependent on endogenous H_2S. It has been reported that adenosine receptor activation (Philipp et al., 2006) or prolonged transient

acidosis during IPostC is able to induce Akt phosphorylation. These signaling mechanisms may act in a concerted manner to trigger Akt activation and its downstream target eNOS to protect heart against ischemia reperfusion injury via inhibiting opening of mitochondrial permeability transition pore (Tsang et al., 2004).

In the present study, two IPostC protocols commonly used by other groups (Lu et al., 2006; Tissier et al., 2007) were employed to investigate whether postconditioning with exogenous H_2S can also produce cardioprotection. Both protocols of H_2S postconditioning produced significant and similar cardioprotective effects on both cardiodynamic performance and myocardial infarction. These data suggest that interference of the early phase of reperfusion may produce strong cardioprotection against ischemic injury. Interestingly, the signaling mechanisms for the cardioprotection induced by the two protocols are not totally same. In addition to the common signaling mechanisms that both SPostC and SPostC2 stimulated Akt and PKCδ, each of them activated different individual signaling mechanisms. SPostC, the six intermittent administrations of H_2S, was able to activate PKCα and PKCε, whereas SPostC2, continuous infusion of H_2S for two min, initiated eNOS phosphorylation. These data suggest that the signaling mechanisms for postconditioning are complicated. Properly manipulation of the early phase of postconditioning may have important clinical implications.

Over the decades, Akt pathway has become such a target due to its role as a signaling pathway where modulation of substrates prevents apoptosis. Akt functions as a survival kinase by phosphorylating a number of apoptosis-regulatory molecules such as BAD, forkhead transcription factors, caspase 9, and IkB kinase to regulate NF-kB

and GSK-3β (Mullonkal and Toledo-Pereyra, 2007). Notably, several studies have suggested that H_2S may protect hearts from myocytes apoptosis (Hu et al., 2007c; Sodha et al., 2008), which could be correlated with our data that SPostC/SPostC2-induced Akt activation. Therefore, the anti-apoptotic effect of H_2S may contribute significantly to the cardioprotection exerted by SPostC/SPostC2 through Akt activation.

Although in the present study I demonstrated that the cardioprotection induced by IPostC and SPostC involves Akt, eNOS and PKC pathways, the effectors of this postconditioning is still unknown. There is a general trend which suggests that mitochondrial K_{ATP} channel opening is one of the downstream effectors of IPostC (Mykytenko, 2005; Penna et al., 2007; Yang et al., 2004b). Previous studies have shown that blockade of K_{ATP} channels attenuated the protective effect of H_2S preconditioning (Bian et al., 2006; Pan et al., 2006). More importantly, H_2S was shown to be a direct K_{ATP} channel opener in vascular smooth muscle cells (Tang et al., 2005; Zhao et al., 2001) and insulin secreting cells (Yang et al., 2005a). As such, the opening of K_{ATP} channels by H_2S could possibly serve as one of the downstream effectors of H_2S postconditioning. In addition, H_2S has been shown to scavenge H_2O_2 in vitro (Geng et al., 2004a) therefore to protect neuronal cells from oxidative stress (Kimura and Kimura, 2004) and hearts from ischemia injuries (Geng et al., 2004a). Moreover, H_2S has been shown to attenuates myocardial ischemia-reperfusion injury by preserving mitochondrial function (Elrod et al., 2007). In this context, the large amount of release H_2S during IPostC may protect the heart from ischemia-reperfusion injury partly via inhibiting reactive oxygen species (ROS) generation (Sun et al., 2005), oxidant

mediated injury (Kin et al., 2004) and preservation of mitochondrial function (Elrod et al., 2007), all of which may also contribute to the effects of SPostC and SPostC2.

In the current study, I used constant flow in our isolated heart model. At the beginning of reperfusion, coronary perfusion pressure increases less and slower in the constant–flow model as compared to constant–pressure model which reaches the selected/baseline value immediately (Penna et al., 2006). Unlike the constant pressure model, this prevents the reactive hyperemia due to sudden increase in pressure that occurs during reperfusion after the global ischemia. This would result in lower production of ROS and eventually resulted in less myocardial injuries (Sato et al., 1997). Indeed, the IPostC showed greater infarct-sparing effect in the constant-flow than in the constant-pressure model (Penna et al., 2006). Therefore, it would be better to test the involvement of ROS in the cardioprotection of H_2S postconditioning using a constant-flow isolated heart model.

I previously demonstrated that H_2S may induce intracellular acidification in aorta smooth muscle cells (Lee et al., 2007). However, the intracellular pH regulatory effect may not play a major role in the cardioprotection induced by SPostC and SPostC2 in the current study. This is because H_2S induced intracellular acidification can only be achieved after administration of H_2S for 5 min (Lee et al., 2007), which is much longer than the perfusion period of H_2S used in the current study.

It was demonstrated in Chapter 3 that endogenous H_2S may participate ischemic preconditioning (IP) as application of PAG could essentially abrogate the protective effect of IPC. This is alike to the observation obtained in the case of IPostC. Similarly, exogenous H_2S could serve as a pharmacological agent to induce IP (H_2S-

preconditioning) in both immediate (Bian et al., 2006) and delayed (Pan et al., 2006; Pan et al., 2007) window. The mechanisms involved have been intensively studied by several groups, including opening of sarcolemmal K_{ATP} channels, PKCs (δ-, α-, ε-isoforms) activation, NOS activation (Bian et al., 2006; Pan et al., 2007), heat shock protein 71 (Bliksoen et al., 2008), ERK and PI3K/Akt pathways (Hu et al., 2008b). In the present study, I showed that PKC, PI3K/Akt, and eNOS are involved in H_2S-postconditioning, indicating that the mechanisms are highly similar to those stimulated by H_2S-preconditioning.

Taken together, it was demonstrated for the first time that endogenous H_2S plays an important role in modulating the cardioprotective effects of IPostC. Postconditioning with exogenous H_2S is able to resume heart contractile functions to a similar extend as those produced by IPostC probably via activation of PKC and/or Akt/eNOS pathway. The effect of H_2S postconditioning against ischemia-reperfusion injury has provided a firm ground to support H_2S as a cardioprotective gasotransmitter.

Chapter 5 Hydrogen sulfide interacts with nitric oxide in the heart - Possible Involvement of nitroxyl

5.1. Introduction

In previous chapter, I found that H_2S increased nitric oxide (NO) production via activation of endothelial NO synthase (eNOS). It would be interesting to find out what is the physiological function of this increased NO together with H_2S administration.

I was also interested to examine the interaction between H_2S and NO in biological system. Several reports demonstrated that H_2S and NO may influence the production of each other (Li et al., 2009; Minamishima et al., 2009; Pan et al., 2006; Yong et al., 2008a; Zhao et al., 2001). In addition, Whiteman et al proposed that H_2S may interact with NO to form an unidentified nitrosothiol moiety, which may regulate the physiological effects of both NO and H_2S (Whiteman et al., 2006).

Bearing the strong reducing capability of H_2S (Szabo, 2007; Wang, 2002; Warenycia et al., 1989) in mind, it is possible that H_2S may putatively reduce NO, with or without the action of enzymes or other biological substances, to form nitroxyl (HNO), the one-electron reduced and protonated sibling of NO. The cardiovascular functions of HNO have been recently studied. It induces vasodilation and enhances heart contractility (Irvine et al., 2008). The potent-positive inotropic effect of HNO appears to be unique among all nitrogen oxides and its property of being unrelated to beta-adrenergic signaling renders HNO to be a tremendously potential pharmacological treatment for heart failure and other cardiovascular ailments. However, HNO has not yet been evidently shown to be endogenously biosynthesized as a signalling molecule. It also remains unclear whether HNO formation can have a specific physiological

function. In the current study, I aim to elucidate the function of the interaction between H_2S and NO, and to explore the plausibility of these two biological gases being a source of endogenous HNO production in the heart.

5.2. Materials and methods

5.2.1. Methods

Isolation of adult rat cardiomyocytes, measurement of intracellular calcium, contractile and relaxation function of myocytes have been described in the Materials and Methods in Chapter 2 and 4 respectively.

5.2.2. Drugs and chemicals

Type 1 collagenase, protease XIV, isoproterenol (ISO), sodium hydrogen sulfide (NaHS), sodium nitroprusside dihydrate (SNP), L-arginine (L-arg), diethylamine NONOate sodium salt hydrate (DEA/NO), Rp-Adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt hydrate (Rp-cAMP), 8-(4-Chlorophenylthio)-guanosine 3′,5′-cyclic monophosphorothioate, Rp Isomer triethylammonium salt (Rp-cGMP), N-acetyl-cysteine, L-cysteine, glutathione (GSH), and caffeine were purchased from Sigma Aldrich, Singapore. Fura-2 was purchased from Molecular Probes Inc. USA. Angeli's salt was purchased from Cayman Chemical, USA. All are dissolved in water, with the exception of Fura-2 (in DMSO) and Angeli's salt (in 0.01M NaOH).

5.2.3. Statistical Analysis

Values presented are mean ± standard error of mean. SPSS for Windows (11.5.0, LEAD technologies, USA) was used to perform the analysis. One-way analysis of variance (ANOVA) was used to determine the difference among groups. A post hoc Bonferroni analysis was used to determine the significant differences between the groups. The significance level was set at $P<0.05$

5.3. Results

5.3.1. Effect of NO increasing agents on cardiomyocyte contraction in the presence or absence of NaHS

Freshly isolated adult rat mycocytes were exposed to 50 μM of each of the following drugs: NaHS (an H_2S donor), L-arg (a substrate of NOS to produce NO), SNP (an NO donor) or DEA/NO (an NO donor). As shown in Figure 5-1A-B, all the three NO increasing agents (L-arg, SNP and DEA/NO) significantly decreased the twitch amplitudes of myocytes. Surprisingly, when given together with NaHS, which itself had no significant effect on myocyte contraction, these three NO increasing agents produced positive, instead of negative, inotropic effect. These data imply that an interaction between NO and H_2S may exist.

Since the three NO increasing agents produced similar effects, and the fact that SNP allows better definition of the NO concentration than L-arg and is much more stable than DEA/NO in aqueous solution(Kowaluk et al., 1992), SNP was chosen for the subsequent experiments. The concentration-dependent effect of the mixture of SNP and NaHS (SNP+NaHS) was observed. As shown in Figure 5-1C, SNP+NaHS (concentration ratio: 1:1) increased myocytes contractility when each concentration was at a range from 50-200 μM. The maximum effect (~150% augmentation) was observed when each concentration was at 100 μM. To avoid possible toxic effect, SNP+NaHS at 50:50 μM was chosen in the subsequent experiments.

Since it was reported that 1 μM SNAP significantly increased cell shortening,(Vila-Petroff et al., 1999) it is interesting to examine whether NaHS+SNP at 50:1 μM could produce stronger effect than they do at 50:50 μM. As shown in Figure 5-

1D, SNP at 1 μM increased myocyte contractility by ~ 28%, but this effect was abolished by co-application with 50 μM NaHS. This data suggest that the concentration ratio of SNP:NaHS is critical to exert the positive inotropic effect.

I further observed whether SNP+NaHS can change the velocities of myocyte contraction and relaxation. As shown in Figure 5-1E, NaS also reversed the maximal velocities of cell shortening (-dL/dt) and cell relaxing (+dL/dt) from negative value caused by SNP alone to the positive values (Figure 5-1E). These data suggest that NaHS+SNP increases the efficiencies of both cell contraction and relaxation.

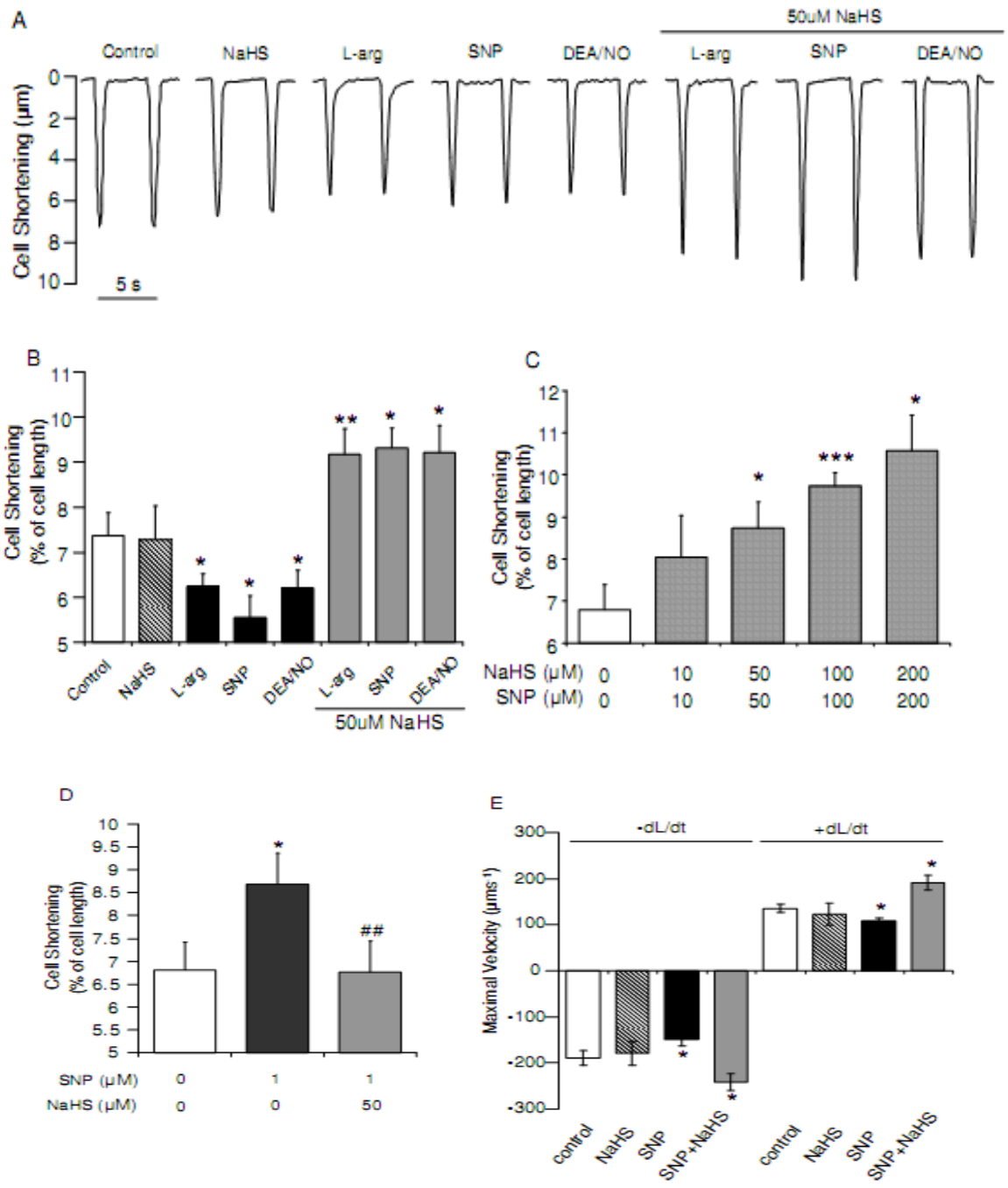


Figure 5-1 Effect of NO increasing agents on myocyte contractility in the presence or absence of NaHS in electrically-stimulated rat ventricular myocytes. (A) Representative tracings of cell shortening in ventricular myocytes treated with L-arginine (L-arg), SNP or DEA/NO in the presence or absence of NaHS. (B) Group data showing that all the three NO increasing agents (L-arg, SNP, and DEA/NO), which themselves alone decreased myocyte contraction, produced positive inotropic effects in the presence of NaHS. (C) Concentration-dependent response of NaHS+SNP (concentration ratio at 1:1, each at 10 ~ 200 μM) on cell shortening. (D) Effect of SNP (1 μM) in the presence or absence of NaHS (50 μM). (E) Effect of NaHS and SNP on the velocity of cell shortening (-dL/dt) and relaxing (+dL/dt). Mean±SEM, n = 7-8, *p<0.05, **p<0.01, ***p<0.001 vs control group, $^{\#\#}$p<0.01 vs SNP+NaHS group.

5.3.2. Effect of SNP on intracellular calcium transients in the electrically-induced (EI) ventricular myocytes in the presence or absence of NaHS

To determine the effect of NO and H_2S donors on calcium handling, I observed the effect of H_2S on the amplitudes of EI-$[Ca^{2+}]_i$ transients. As shown in Figure 5-2A-C, NaHS had no significant effect, but SNP decreased the amplitudes of EI-$[Ca^{2+}]_i$ transients. However, SNP+NaHS significantly augmented the amplitudes of calcium transients (Figure 5-2A-C). I also analyzed the decay time constant, Tau. As shown in Figure 5-2D, NaHS+SNP evidently shortened the decay time when compared to control, NaHS or SNP alone, suggesting that NaHS+SNP may induce a faster calcium removal from cytosol via increasing the activity of either sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), or Na^{+}-Ca^{2+} exchanger.

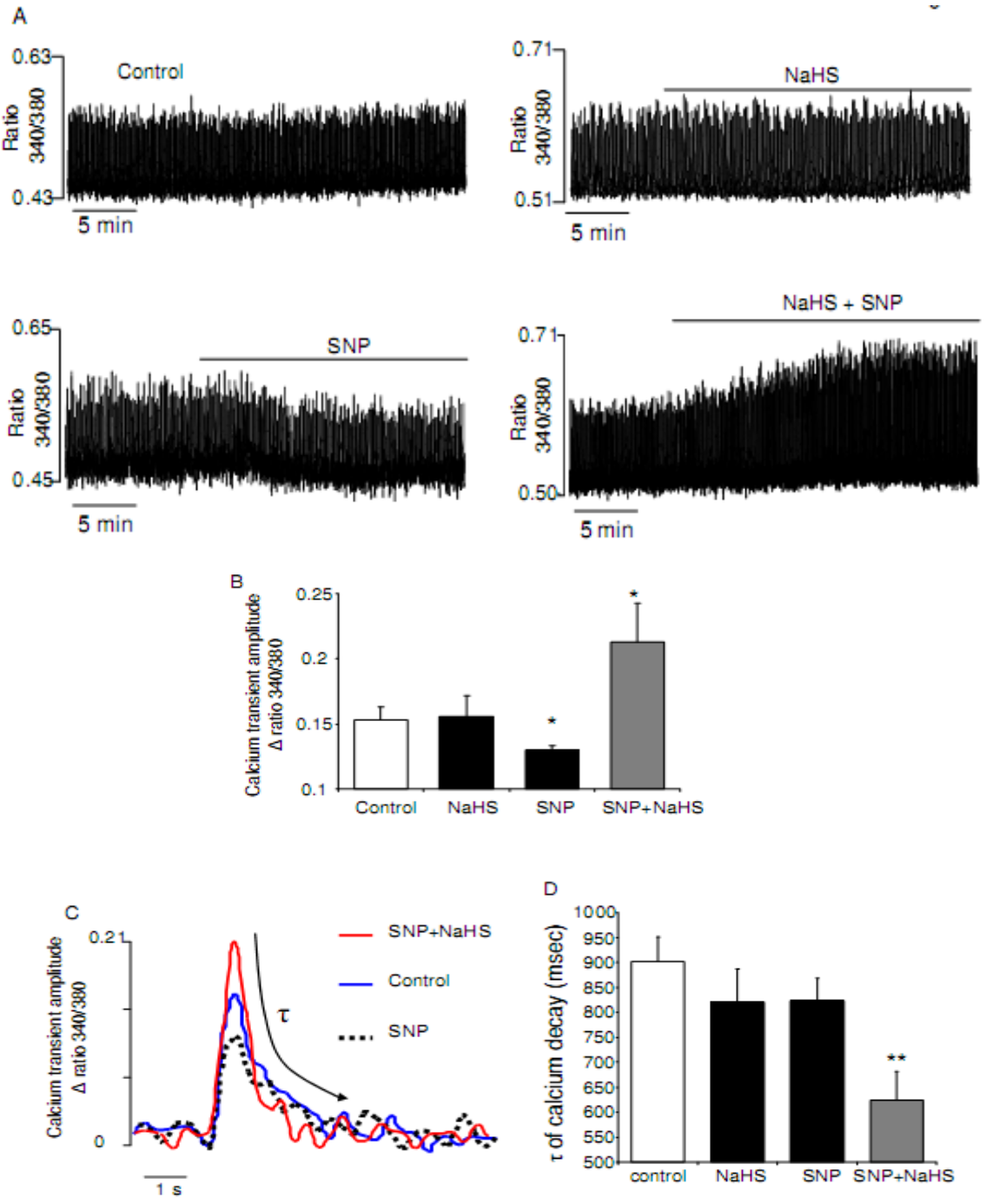
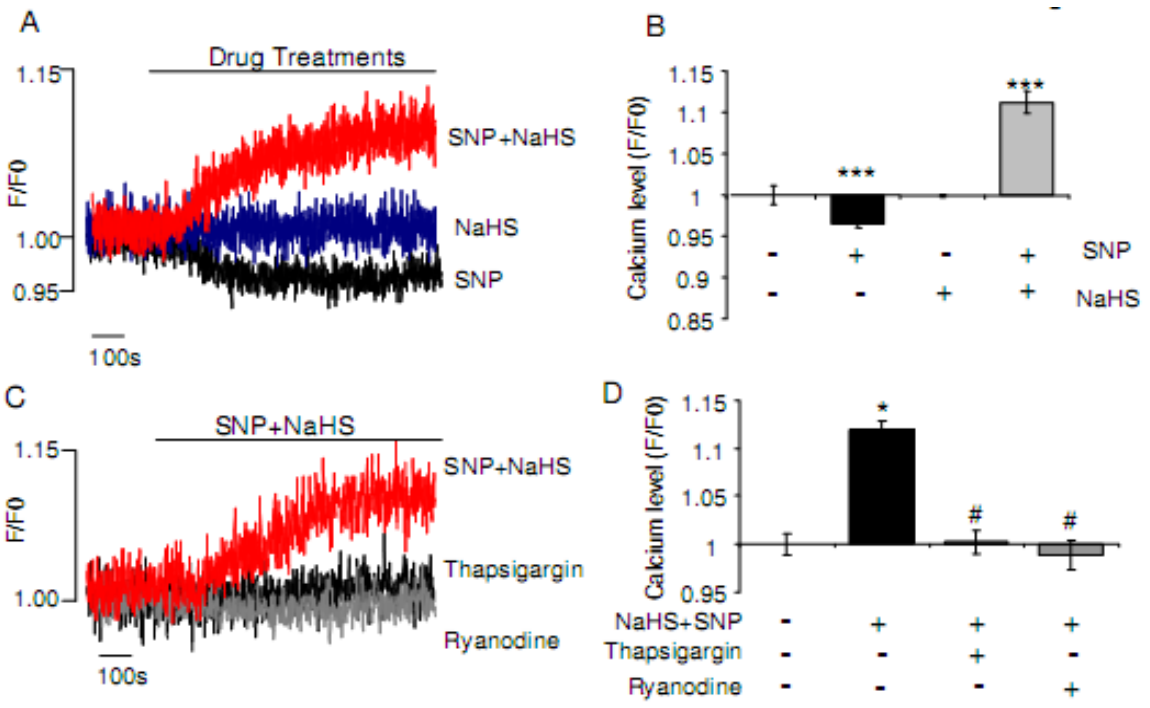


Figure 5-2 Effect of SNP on EI-$[Ca^{2+}]_i$ transients in the presence or absence of NaHS in the rat ventricular myocytes. (A-B) Representative tracings (A) and group data (B) showing that NaHS+SNP increased, whereas SNP alone decreased the amplitudes of EI-$[Ca^{2+}]_i$ transients. (C-D) Representative tracings (C) and group data (D) showing that NaHS+SNP increased the velocity of calcium decay as shown by the decreased decay time. Mean±SEM, n = 6-9, *p<0.05, ** p<0.01 vs control group.

5.3.3. Effect of SNP on resting calcium and caffeine-induced calcium transients in the ventricular myocytes in the presence or absence of NaHS

Figure 5-3A and 5-3B show that SNP (50 μM) significantly decreased the calcium level at resting status. However, SNP markedly increased the resting calcium level in the presence of NaHS, which itself alone had no significant effect on calcium level. I also found that the effect of NaHS+SNP on resting calcium was dependent on intracellular store, as pretreated the myocytes with thapsigargin (2 μM, 1 hr) or ryanodine (15 μM, 1 hr) essentially abolished the effect of NaHS+SNP. (Figure 5-3C & 3D) Interestingly, SNP+NaHS failed to affect the amplitude and the decay velocity of caffeine-induced calcium transients (Figure 5-3E-H). These data suggest that the inotropy and lusitropy of SNP+NaHS may be related to ryanodine receptor and SERCA, but not via altering the sarcoplasmic reticulum (SR) calcium content and the function of Na^{+}-Ca^{2+} exchanger.



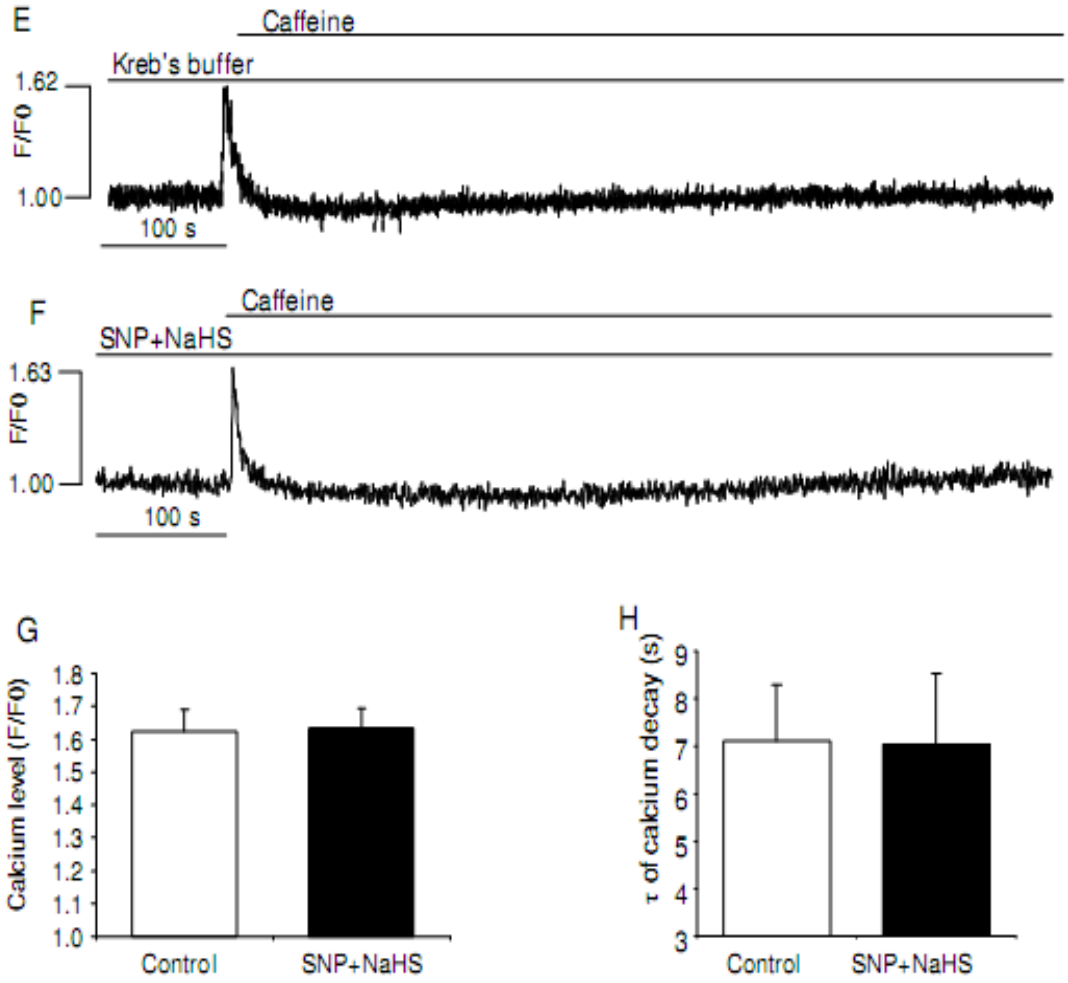
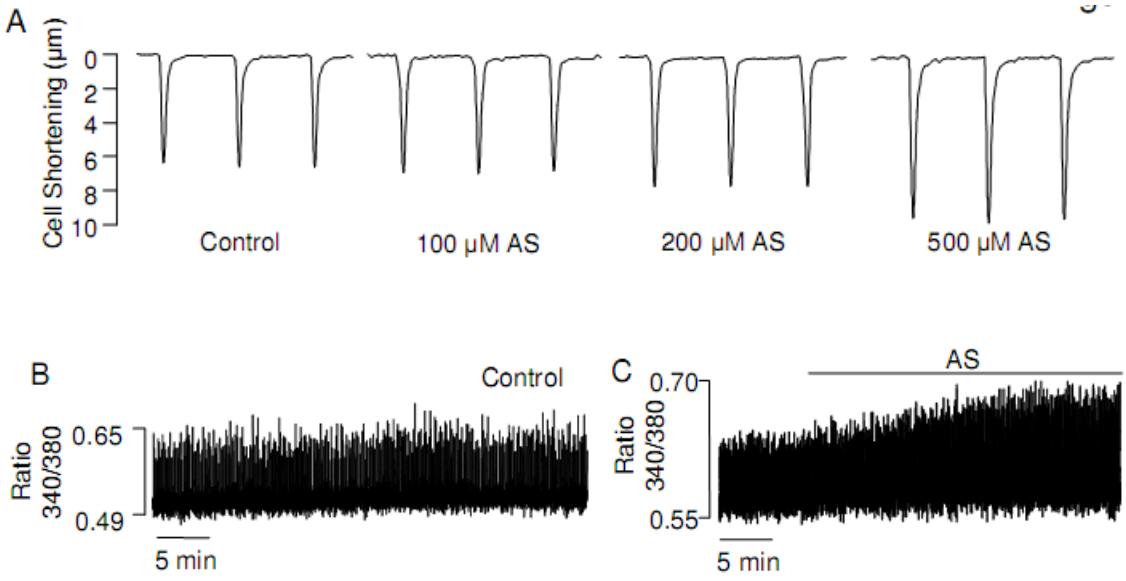


Figure 5-3 Effect of SNP on resting $[Ca^{2+}]_i$ and caffeine-induced $[Ca^{2+}]_i$ transients in the rat ventricular myocytes. (A) Representative tracings of resting $[Ca^{2+}]_i$ in myocytes treated with NaHS, SNP or SNP+NaHS. (B) Group data showing that SNP+NaHS increased resting $[Ca^{2+}]_i$ level when it was measured 1000 s after drug treatment. (C-D) Representative tracings (C) and group data (D) showing that the effect of NaHS+SNP was sensitive to ryanodine and thapsigargin. (E-F) Representative tracings of caffeine-induced $[Ca^{2+}]_i$ transients in the absence (E) or presence (F) of SNP+NaHS. (G-H) Group data showing that SNP+NaHS treatment had no significant effect on both amplitudes and decay of caffeine-induced $[Ca^{2+}]_i$ transients. Mean±SEM, n = 6-9, * $p<0.05$, ***$p<0.001$ vs control, $^{\#}p<0.05$ vs SNP+NaHS group.

Effect of HNO on cell contractility, EI-$[Ca^{2+}]_i$ transients and resting calcium in isolated cardiomyocytes

The above data suggest a new compound may be formed upon mixing NO and H_2S. To examine the involvement of HNO, Angeli's salt (AS), an HNO donor, was used. As shown in Figure 5-4, AS concentration-dependently augmented the amplitudes of both cell shortening (Figure 5-4A & 5-4D) and $[Ca^{2+}]_i$ transients in electrically stimulated ventricular myocytes (Figure 5-4B, C & F). The effect of AS was completely abolished when the cells were pre-incubated with HNO scavenger N-acetyl-cysteine (NAC, 1 mM)(Favaloro and Kemp-Harper, 2007), L-cysteine (L-cys, 1 mM)(Irvine et al., 2008) and glutathione (GSH, 1 mM)(Tocchetti et al., 2007) (Figure 5-4E & G). This is in line with several previous reports(Favaloro and Kemp-Harper, 2007; Irvine et al., 2007; Paolocci et al., 2001; Tocchetti et al., 2007). In addition, I also found that AS at 500 µM increased resting calcium by ~10%, which was abolished by pretreatment of the cells with thapsigargin (2 µM, 1 hr) or ryanodine (15 µM, 1 hr) (Figure 5-4H & I). These data further suggest that AS/HNO and NaHS+SNP may act on the similar sites.



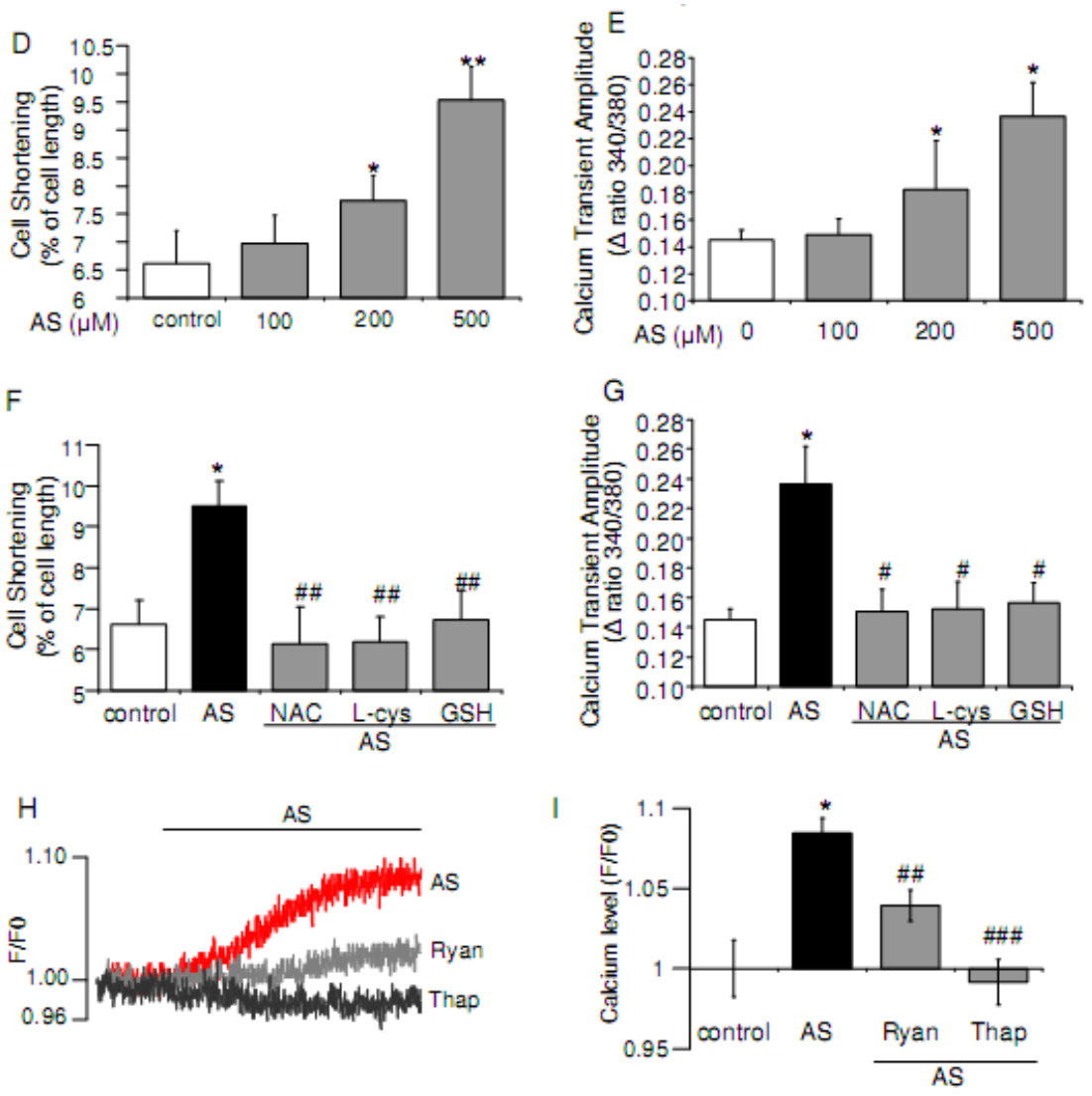
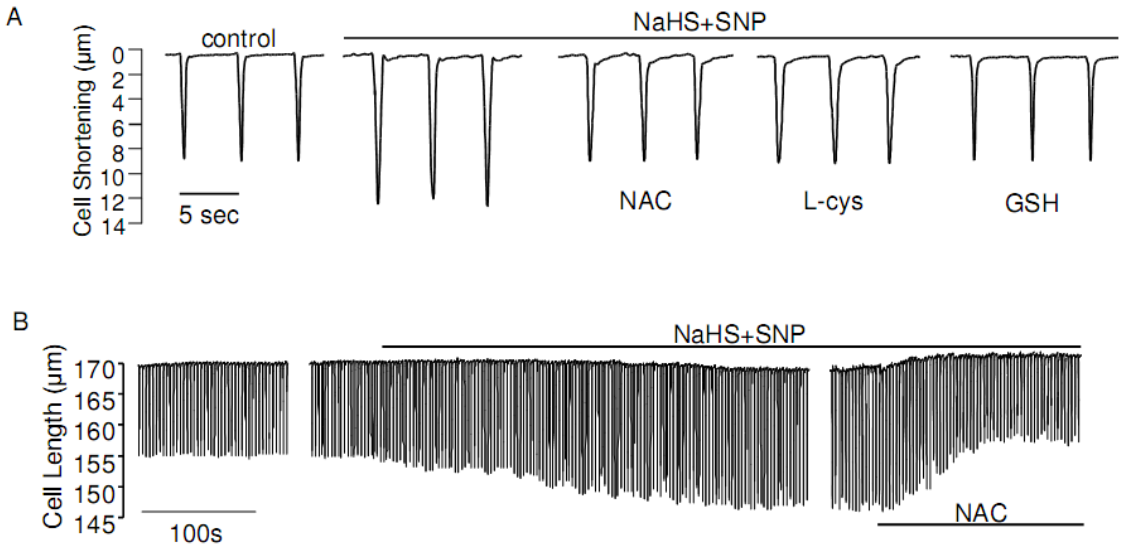


Figure 5-4 Effect of AS on cell shortening in electrically-stimulated rat ventricular myocytes in the absence or presence of HNO scavengers. (A-C) Representative tracings of myocyte shortening (A) and $[Ca^{2+}]_i$ transients (B-C) in the presence or absence of AS. (D-E) Concentration-dependent effect of AS on cell shortening (D) and $[Ca^{2+}]_i$ transients (E). (F-G) HNO scavengers (NAC, L-cys and GSH) abolished the effect of AS (500 μM) on cell shortening (F) and $[Ca^{2+}]_i$ transients (G). (H-I) Representative tracings and group data showing that the effect of AS was sensitive to ryanodine (Ryan) and thapsigargin (Thap). Mean±SEM, n = 5-9, *p<0.05, ***p<0.001 vs control, $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 vs AS alone group.

5.3.4. Effect of NO+H_2S involves HNO

To test the hypothesis that the effect of NO+H_2S is due to the formation of HNO, I incubated the cells with the different HNO scavengers 10 min prior to the application of SNP+NaHS. Our data showed that all the three scavengers (NAC, L-csy and GSH) abolished the effect of SNP+NaHS on both cell shortening (Figure 5-5A & D) and $[Ca^{2+}]_i$ transients (Figure 5-5C & E). Similarly, addition of NAC (1 mM) 20 min after administration of SNP+NaHS reversed the positive inotropic effect of SNP+NaHS (Figure 5-5B). These data suggest that HNO may mediate the effect of NO+H_2S.



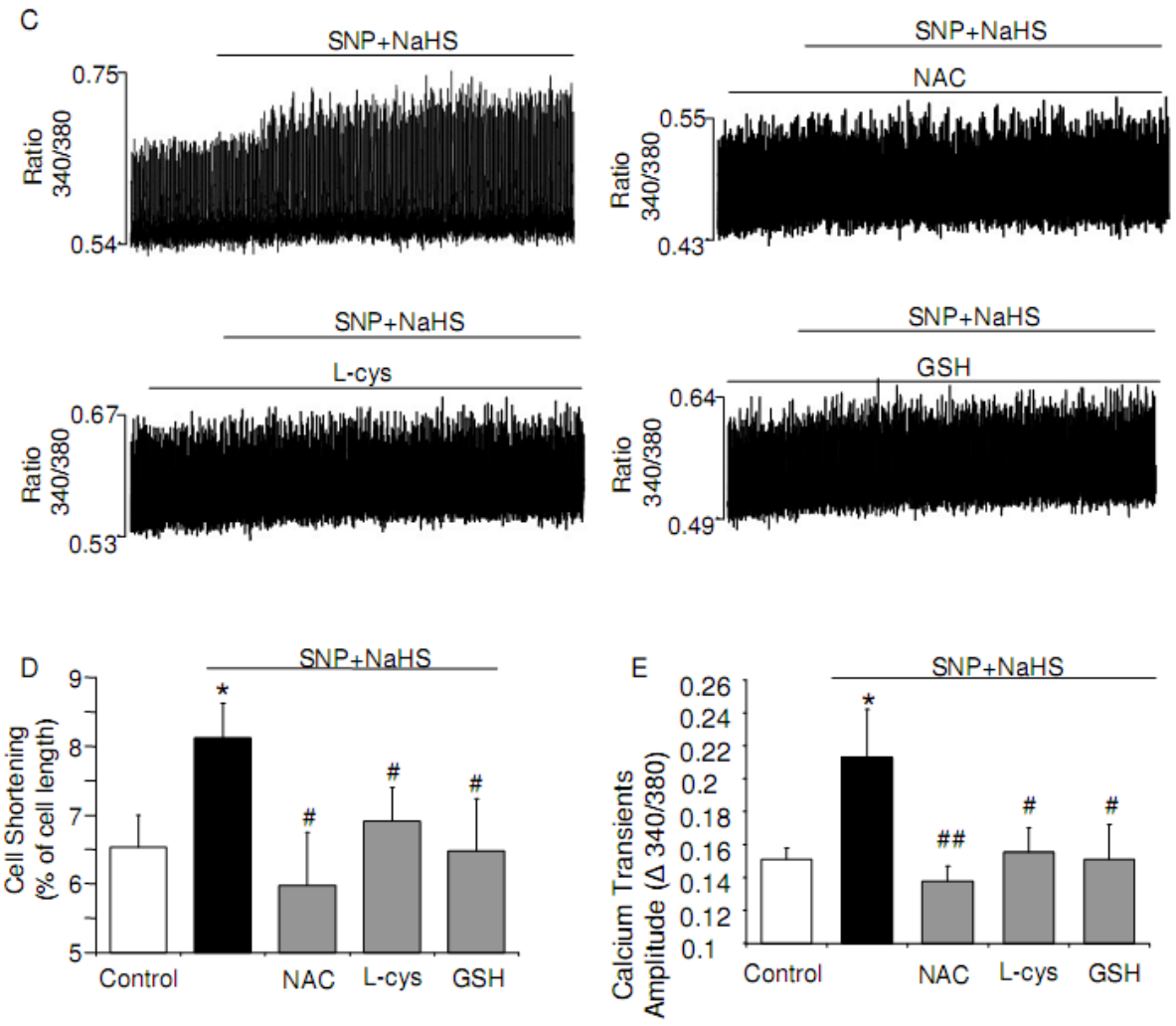
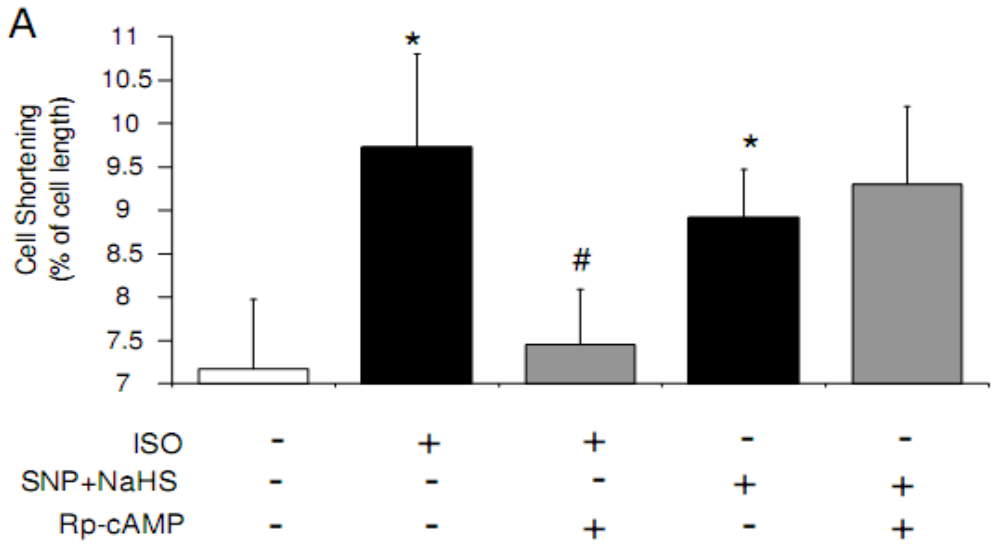


Figure 5-5 Effect of NaHS+SNP on myocyte contraction and EI-$[Ca^{2+}]_i$ transients in the presence or absence of HNO scavengers. (A & C) Representative tracings of myocytes cell shortening (A) and $[Ca^{2+}]_i$ transients (C) in the control and NaHS+SNP treatment groups in the cells pre-incubated with or without a HNO scavenger (NAC, L-cys or GSH). (B) Representative tracing of continuous recording of myocytes cell shortening showing that NAC reversed the positive inotropic effect of NaHS+SNP. (D-E) The effects of NaHS+SNP on cell shortening (D) and $[Ca^{2+}]_i$ transients (E) were abolished by pretreatment with HNO scavengers. Mean±SEM, n = 8-12, *$p<0.05$ vs control, $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs NaHS+SNP group.

5.3.5. The positive inotropic effect of H_2S+NO is independent of cAMP/PKA and cGMP/PKG pathways

cAMP is an important second messenger to regulate heart contractility. To examine whether the effect of H_2S+NO involves cAMP, I pretreated the ventricular myocytes with Rp-cAMP (100 μM), a membrane permeable PKA inhibitor, 5 min before the addition of NaHS+SNP. Figure 5-6A shows that Rp-cAMP significantly attenuated the positive inotropic effect of ISO, but had no effect on that of SNP+NaHS. Similarly, Rp-cGMP (10 μM, a PKG inhibitor) abolished the negative inotropic effect of SNP (500 μM), but failed to affect the positive effect of SNP+NaHS (Figure 5-6B). These data suggest that the effect of H_2S+NO is also independent of cGMP/PKG pathway.

Both ISO (10 nM) and SNP+NaHS induced positive inotropic effects (34.6 ± 8.6% and 26.6 ± 1.9%, respectively, Figure 5-6C). Of note, co-application of ISO and SNP+NaHS produced even stronger contraction (62.4 ± 6.4%, Figure 5-6C). The above data suggest that the action of H_2S+NO may be in parallel with the β-adrenergic pathway, and notably, additive to β-adrenoceptor stimulation.



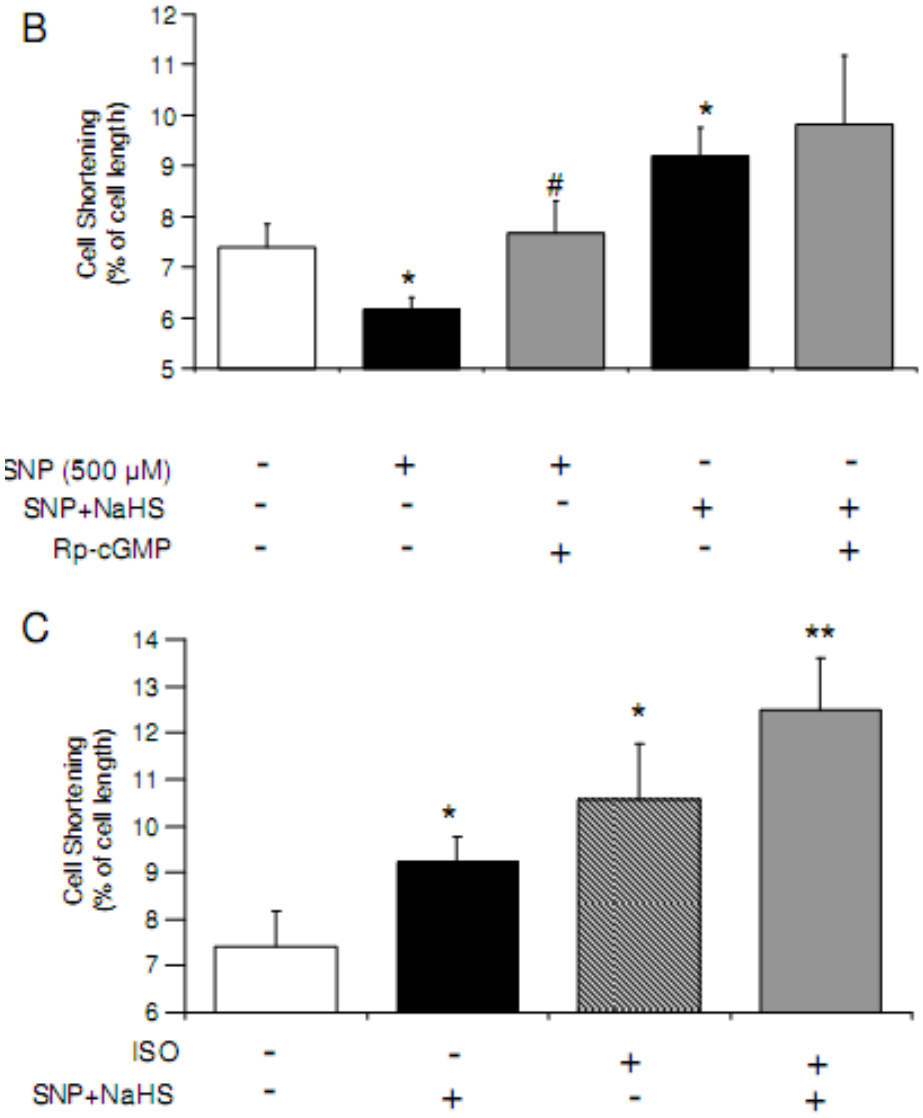


Figure 5-6 Effect of NaHS+SNP on myocyte contraction upon blockade of PKA or PKG or stimulation of β-adrenoceptor. (A) Blockade of PKA with Rp-cAMP significantly attenuated the effect of ISO, but not that of NaHS+SNP, on myocyte contraction. n = 8-12. (B) Blockade of PKG with Rp-cGMP significantly attenuated the effect of SNP (500 μM), but that of NaHS+SNP, on myocyte contractility. n = 6-11. (C) Additive effect of SNP+NaHS and ISO on myocyte contraction, n= 11-15. Mean±SEM, *$p<0.05$, **$p<0.01$ vs control; $^{\#}p<0.05$ vs ISO or SNP alone.

5.4. Discussion

The main objective of this study is to investigate the interaction between H_2S and NO in the heart. By measuring myocyte contractility and EI-$[Ca^{2+}]_i$ transients, I found that NaHS at 50 μM had negligible effect, whereas NO donors produced negative inotropic effect in the cardiomyocytes. This is consistent with the previous reports (Flesch et al., 1997; Miranda et al., 2005; Sun et al., 2008; Tatsumi et al., 2000; Yong et al., 2008a; Yong et al., 2008b). However, unexpectedly, when these two kinds of donors mixed together, I observed a marked increase in the resting calcium, EI-$[Ca^{2+}]_i$ transients and myocyte contractility accompanied by augmented velocities of myocyte contraction and relaxation. Since both stimulating endogenous NO production with L-arginine and exogenous application of NO donors produced similar effects, the possibility that the effect merely resulted from chemical reaction of two donors can be excluded. The completely opposite effects of NaHS+SNP as compared to that caused by each individual donor led us to hypothesize that a new compound is formed from the interaction between H_2S and NO.

The importance of the reducing capability of H_2S (Szabo, 2007; Wang, 2002; Warenycia et al., 1989) and the structural property of HNO with one-electron reduced and protonated sibling of NO prompted us to postulate HNO as one of the most likely candidates of the new compound formed during the interaction of H_2S with NO. To test this hypothesis, I determined the effect of AS, an HNO donor, and different types of thiols (NAC, L-cys and GSH), which were shown to be effective HNO scavenger (Irvine et al., 2008; Paolocci et al., 2007), on myocyte function. I found that AS mimicked the effect of SNP+NaHS, whereas the thiols abolished the effects of both AS

and NaHS+SNP. Our data suggest that the cardiac effect of NaHS+SNP may involve HNO.

Previous studies demonstrated that HNO donors improve function in the failing heart via β-adrenoceptor independent pathway(Paolocci et al., 2003; Paolocci et al., 2001). Furthermore, it was reported that inhibition of cAMP/PKA and cGMP/PKG had no impact on AS inotropy (Tocchetti et al., 2007). Similarly, in the present study I found that the action of NaHS+SNP was independent of cAMP/PKA and cGMP/PKG pathways. In addition, HNO and β-adrenoceptor stimulation produced additive positive inotropic effects whereas both NO donors (Massion et al., 2005; Tocchetti et al., 2007) and NaHS (Yong et al., 2008b) negatively modulate β-adrenergic stimulation. These features of NaHS+SNP have laid further supports to the hypothesis that HNO may mediate the effect of NaHS and SNP.

HNO enhances fractional calcium release from SR via increasing the open probability of ryadonie receptors (Tocchetti et al., 2007). This explains why SNP+NaHS increased the amplitudes of myocyte contraction and $[Ca^{2+}]_i$ transients caused by electrical stimulation. Our data that the elevated resting $[Ca^{2+}]_i$ was abolished by ryanodine further confirm that SNP+NaHS may also act on ryanodine receptors.

HNO also activates SERCA through S-glutathiolation at cysteine 674 (Lancel et al., 2009). This is supported by our data that SNP+NaHS accelerated the decay rate of EI-$[Ca^{2+}]_i$. Interestingly, HNO did not affect the total calcium uptake to SR, although it significantly increased calcium uptake velocity (Tocchetti et al., 2007). This helps to explain why SNP+NaHS improved SERCA activity without altering SR calcium load.

So how is HNO produced in the biological system? H_2S may interact with NO via direct or indirect ways. As a strong reducing agent, H_2S might interact directly with NO to form HNO. HNO may also be generated via reduction of NO by elements of the electron transport system in mitochondria (Zhao et al., 1995), ubiquinol(Poderoso et al., 1999), manganese superoxide dismutase (Niketic et al., 1999), and xanthine oxidase (Saleem and Ohshima, 2004). In addition, it was also demonstrated that HNO can be generated from NOS especially in the absence of tetrahydrobiopterin (Fukuto et al., 2005a), or after oxidation of the NOS intermediates, N-hydroxy-L-arginine(Fukuto et al., 2005a; Irvine et al., 2008) and hydroxylamine (Donzelli et al., 2008). Notably, several other reports also demonstrated that H_2S may enhance eNOS phosphorylation (Minamishima et al., 2009; Pan et al., 2006; Yong et al., 2008a). It is therefore possible that HNO can be generated indirectly from H_2S-stimulated NOS activation. Furthermore, the reaction of S-nitrosothiol with excess thiols may also result in release of HNO. (Paolocci et al., 2007; Spencer et al., 2003) Whiteman *et al.* reported that a biologically inactive nitrosothiol may be chemically formed from the reaction between H_2S and NO (Whiteman et al., 2006). These findings suggest that the intermediate substance, nitrosothiol, may further react with thiols to form HNO (Minamishima et al., 2009; Pan et al., 2006; Yong et al., 2008a).

we and other groups have demonstrated that eNOS or nNOS activation is important for H_2S to exert cardioprotective effects. For instances, H_2S may be used to induce pharmacological pre- (Pan et al., 2006) and post-conditioning(Yong et al., 2008a) to protect the heart against ischemia-reperfusion injury via several pathways including eNOS activation. Minamishima *et al.* also found that administration of H_2S

donor markedly improves myocardial function and survival after cardiac arrest in mice, but the protective effects of H_2S was abrogated in eNOS deficient mice (Minamishima et al., 2009). These findings suggest that NO plays an important role in the cardioprotection conferred by H_2S in different pathological models. Given the positive inotropic effect of H_2S+NO, generation of HNO in the reaction of these two gases may be important to resume the depressed cardiac function back to normal after cardiac arrest or ischemia-reperfusion insult.

The biological effect and significance of HNO need to be confirmed by detection of HNO in the interaction of H_2S and NO. Unfortunately, there is no specific and sensitive detection method for HNO measurement. The current indirect detection methods for HNO, including measurement of N_2O, reductive nitroxylation of oxidized metal complexes and detection of reaction products of HNO with thiols, cannot be applied to intact cells and fail to detect low level of HNO production (Irvine et al., 2008). In addition, the high reactivity of both HNO and NO, particularly self consumption by dimerization (Fukuto et al., 2005b), creates extra challenges for HNO detection. The lack of a specific HNO measurement method hampered us in demonstrating the conclusive evidence that HNO is the playmaker of the inotropic effect induced by H_2S+NO. A specific method needs to be developed to test whether HNO is produced by the reaction between H_2S and NO.

Another interesting question to be asked is that whether sufficient H_2S and NO can be produced in the human body to generate HNO during the physiological situation. The rate of H_2S production in tissue homogenates has been reported to be about 1 – 10 pmoles per second per mg protein, resulting in low micromolar extracellular

concentrations (Doeller et al., 2005). Previous study showed that both rat and human plasma contain approximately 50 μM of H_2S (Zhao et al., 2001), which were recently challenged by Whitfield *et al* who found that the free H_2S gas was essentially undetectable (<100 nM total sulfide) in all animals (Whitfield et al., 2008). On the other hand, mammalian plasma nitrite level, which is often used to indicate the NO level due to its relatively stable characteristic, was shown to range from ~100 nM to 1μM (Rassaf et al., 2003). In this context, it is possible that nanomolar of HNO may be constitutively formed within mammalian system. Mice with genetic deletions of both eNOS and nNOS together with CSE will be crucial to find out whether this nanomolar concentration of HNO is enough to exert any significant physiological effect in the cellular microenvironment. This will be of both scientific and clinical importance, as the finding may improve the understanding of cardiac contractile mechanisms.

Perhaps HNO may play a more important role during pathological situations. For instance during inflammation, iNOS is induced and activated in myocardium. This may lead to large production of cytosolic NO. Similarly, it has also been reported that H_2S level is markedly higher during inflammation as compared to physiological level (Li et al., 2005; Mok et al., 2004). This may imply that during inflammation, large amounts of NO and H_2S can be produced and lead to over-production of HNO which may increase $[Ca^{2+}]_i$ and therefore induces arrhythmias. As such, our study may be helpful to reveal a novel therapeutic strategy to treat inflammation-induced arrhythmias.

There are several limitations in this study. The hypothesis that HNO is generated after mixing H_2S and NO is no conclusively proven with direct HNO measurement, thus one should not rule out the possibility that the resultant inotropic effect of H_2S+NO may

be due to the interaction of the two signalling pathways stimulated by H_2S and NO individually. Although the hypothesis that the generation of HNO by H_2S+NO is supported by the data showing the effects of H_2S+NO can be abolished by thiols, we should also bear in mind that the thiols used in this study are not HNO-specific scavengers because they are also able to scavenge reactive oxygen species.

In conclusion, our study shows that H_2S may interact with NO to form a new compound (probably HNO) which produces positive inotropic and lusitropic effect. Our study may add another dimension on the study of gasotransmitters, and potentially change the therapeutic strategy to treat cardiovascular diseases.

Chapter 6 General Discussion

This thesis first investigated the physiological, pathological and therapeutic roles of H_2S in normal or ischemic heart followed by examining the biological and physical interaction of H_2S with NO, another important gaseous mediator. It was found that H_2S regulates β-adrenergic function in the heart and mediates the cardioprotection caused by ischemic preconditioning and postconditioning. By focusing on the effect of H_2S on ECG and contractile function of the isolated heart, as well as cellular calcium handling and contractility in the models of I/R and beta-adrenergic stimulation, our studies evidently demonstrated that H_2S may serve as pharmacological preconditioning & postconditioning agent, and produces potent cardioprotective effects which translated into decreased cell death, prevention of intracellular calcium overload, preserved contractile function, restraint of infarct size, as well as reduction of both severity and duration of ventricular arrhythmias. Further investigations, focusing on the role of H_2S on intracellular NO production, lead to a novel understanding of the complicated functions exerted by these two gases, which may potentially change the therapeutic strategy to treat cardiovascular diseases.

Using isolated rat cardiomyocytes, I identified several essential signaling components and intracellular events mediating the protection afforded by H_2S treatment. With free passage through the plasma membrane, H_2S preconditioning exerts cardioprotective effect via directly or indirectly opening of sarcolemmal K_{ATP} channel, as well as activation of different PKC and NOS isoforms. Via some unclear mechanisms, H_2S post conditioning activates Akt/eNOS pathways and also translocates

different PKC isoforms (PKC-α, -δ, and –ε) from cytosol to membrane fraction of the cells where calcium handling proteins such as SERCA and NCX are located. In addition, H_2S treatment effectively antagonizes β-adrenergic stimulation via inhibiting adenylyl cyclase activity. The effect of H_2S on PKC activation, which results in faster clearing of cytosolic calcium back to SR or extracellular compartment (Pan et al., 2007), together with its effect to reduce cAMP production, which causes less calcium-induced calcium release from SR, make H_2S a powerful therapeutic agent to ameliorate calcium overload in myocytes. This beneficial effect of H_2S may hence contribute to reduced apoptosis/necrosis and hypercontracture of the myocytes, which ultimately lead to decrease in infarct size and preservation of contractile function.

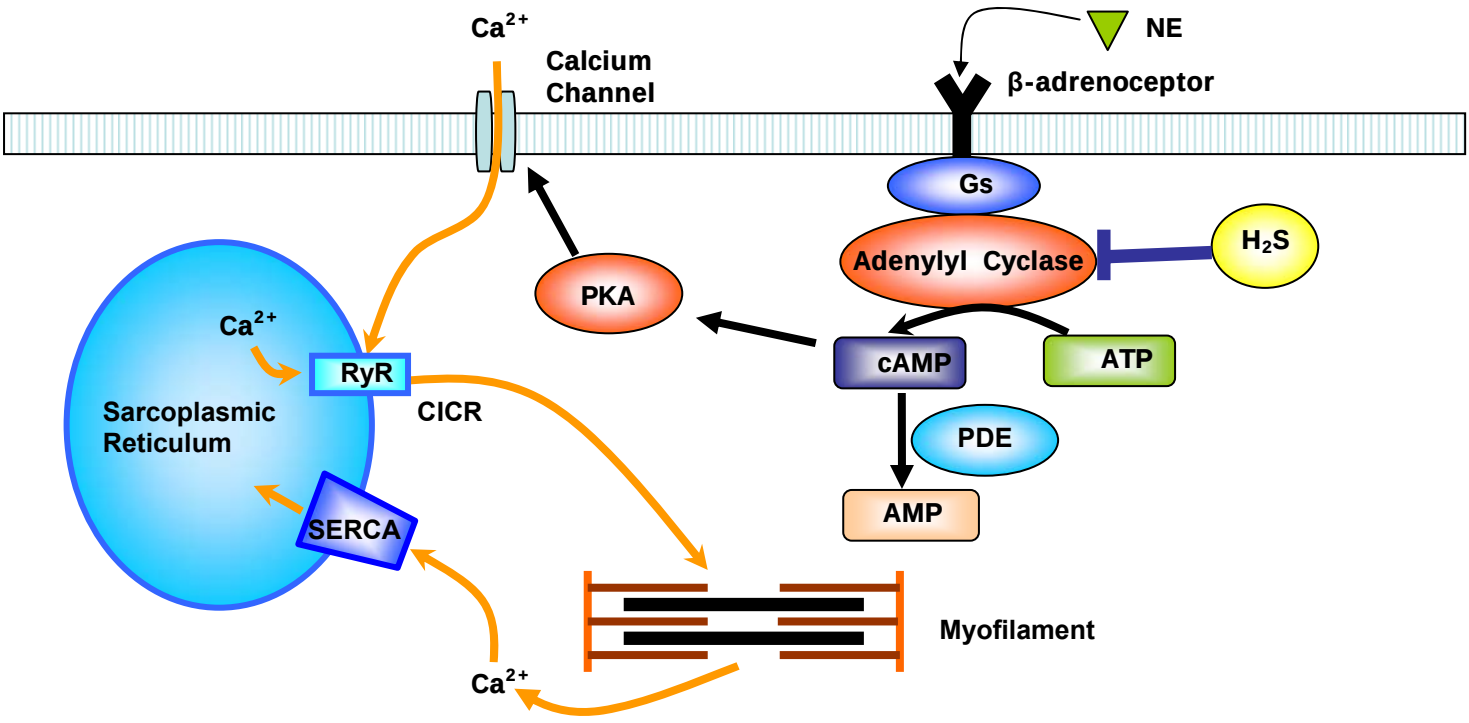


Figure 6-1 H2S negatively regulates β-adrenergic system via inhibiting adenyl l cyclase activity. Gs, stimulatory G protein; PDE, phosphodiesterase; RyR, Ryanodine receptor; PKA, protein kinase A; NE, nor-epinephrine; cAMP. cyclic-adenosine monophosphate; ATP, adenosine triphosphate.

However, I also bear in mind that the whole scenario of the signal transduction could be much more complicated. The signaling pathway outlined above is by no means the only route for H_2S treatment to exert its functions. Of note, our and other groups have shown that the cardioprotective effect of H_2S was significantly contributed by NOS activation (Pan et al., 2006) (Minamishima et al., 2009), in line with these studies, I found that H_2S enhanced eNOS phosphorylation and intracellular NO production in cardiomyocytes, which may in turn react with H_2S and form a new compound endogenously. With the potential involvement of H_2S, NO and their reaction product, which may possess different functions via acting on variety of pathways individually, it is more like a signal network than single pathways which transforms the extracellular stimulus of H_2S into the final protection.

Intriguingly, during the process of investigating the effect of H_2S on NO production, I found that the resultant effect of H_2S+NO is completely opposite to its parental gas respectively (Table 7-1). By comparing the effect of H_2S+NO and HNO on cardiomyocytes functions (Table 7-2), I are convinced that H_2S+NO may form HNO with the help of, for instance, elements of the electron transport system in mitochondria (Clarkson et al., 1995; Zhao et al., 1995). The hypothesis that mixing H_2S with NO may form HNO, would fill up a major knowledge gap in our understanding on why NO, which showed a positive inotropic effect on myocytes and hearts in low concentration, become negative inotropy at high concentrations (Massion et al., 2003). These intriguing observations are not hard to be explained when I consider the existence of basal H_2S production and the interesting observations may be due to the formation of HNO in the experimental system.

Additionally, the understanding of the potential formation of HNO from the reaction between H_2S and NO may be important when both of the gaseous transmitters are significantly up-regulated in some pathological conditions, for examples during inflammation. On the other hand, mixture of H_2S and NO could be an effective treatment to heart failure, since the reaction product of H_2S and NO may improve heart functions via cAMP-independent pathway. In general, my study has highlighted the importance to study the function of gases as a complex rather than individual.

Table 7-1 Comparison of the biological effects of NO, H2S and NO+H2S on the cardiomyocytes

	Biological effect	NO	H_2S	NO+H_2S
1	Inotropy	negative	negative	Positive
2	intracellular calcium	decrease	no change/decrease	Increase
3	Sensitive to thiols?	No	probably No	Yes
4	regulation of β-adrenergic function	Negative	Negative	Additive

Table 7-2 Comparison of the effects of NO, H2S and NO+H2S on the calcium handling machinery in cardiomyocytes

	Biological effect	NO+H_2S	HNO
1	Inotropy	Positive	Positive
2	intracellular calcium	Increase	Increase
3	Sensitive to thiols?	Yes	Yes
4	SERCA activity	Increase	Increase
5	NCX activity	No change	No change
6	SR calcium load	No change	No change
7	regulation of β-adrenergic function	Additive, parallel	Additive, parallel

Ever since the first discovery of endogenously produced H_2S and the expression of the enzymes responsible for its production in the brain, vasculature, liver and heart, a large amount of works have been done to examine the physiological and pathological roles of this new emerging gasotransmitter. However, the majority of the evidence was

based on the exogenous administration of H_2S donor and/or non-specific CSE or CBS inhibitors, which render the physiological role of H_2S in these mammalian systems inconclusive and controversial. As such, study on mice with genetic deletion of H_2S -producing enzymes is of importance. Recently, Yang et al successfully developed this kind of genetically manipulated mice, and further reported that pronounced hypertension and diminished endothelium-dependent vasorelaxation were observed in CSE knock out mice (Yang et al., 2008). This group of researchers found that the relaxation induced by methacholine, an agonist of muscarinic receptor, in the PE-precontracted mesenteric arteries was significantly impaired in $CSE^{-/-}$ mice, suggesting that H_2S functions as an endothelial cell-derived relaxing factor (EDRF) in this vascular bed (Yang et al., 2008). This is in line with the reports mentioned previously, and provides direct evidence that H_2S is a physiologic vasodilator and regulator of blood pressure. It will be of scientifically significance shall more experiments be done on this CSE knock out mice to confirm the observations in my studies.

To date, simple sulfide salts, most commonly NaHS, have been the H_2S-releasing drugs used in most of the study published around the world, including our present study. NaHS is known to release H_2S instantaneously in aqueous solution. Because the release of endogenous H_2S from cells is likely to occur in lesser amounts and at a much slower rate, NaHS may not mimic the biological effects of naturally produced H_2S. Yet, based on findings in the current study, preconditioning or postconditioning with NaHS is able to protection against cardiac ischemia/reperfusion injury, even if it only transiently increases the circulating H_2S levels. When compared to slow H_2S-releasing compounds like GYY4137 (Li et al., 2008) and S-declofenac (Li et al., 2007), NaHS, being a

relatively pure H_2S-donor which only releases Na^+ and HS^- ions, offer an easy definition of H_2S concentration when it is used in different experiments to delineate the biological functions of H_2S.

However, development of organic compounds that slowly release free H_2S over extended periods of time is necessary, because a slower and lower H_2S concentration release to patients is essential in terms of sustained therapeutic effect as well as reduced potential toxicity and side effects of H_2S. Recently there has been increasing interest looking into the feasibility of reducing the side effects of certain drugs by adding H_2S-releasing moiety on the compounds. Administration of H_2S donors could prevent the decrease in gastric blood flow induced by nonsteroidal anti-inflammatory drugs (NSAIDs), as well as attenuating NSAID-induced leukocyte adherence (Fiorucci et al., 2006; Ortiz et al., 2001). Indeed, it was reported that a H_2S-releasing derivative of diclofenac, a kind of NSAID, was substantially better tolerated, in terms of gastric damage, than the parent drugs.(Li et al., 2007; Wallace et al., 2007)

Since long period of H_2S treatment has shown therapeutic effects, drugs like GYY4137 or S-declofenac may be more useful for patients who have been discharged from hospital but require longer term medication to improve the prognosis of the related heart diseases. In addition, this kind of slow H_2S-releasing drugs could be useful for treating patients who fail to access to H_2S preconditioning or postconditioning before or after, respectively, occurrence of myocardial infarction. Bearing the cardioprotective effect in mind, H_2S, on the other hand, may serve as a conjunctive treatment with some other treatments which may cause cardiac toxicity, for examples, anthracyclines, which are very effective antineoplastic agents with a broad antitumor spectrum (Huang et al.,

2010). Production of H_2S-releasing anthracylcines may greatly reduce the cardiac toxicity of the parental drug whereby increase the therapeutic window of this drug and render it more effective against tumors.

Another potential area is the development of H_2S as an inhaled gas or as a parenteral injectable. Inhaled NO has set a precedent for development of medical gases. NO was also first known as a toxic gas and is currently approved for use in infants with primary pulmonary hypertension (Hillier, 2003; Kinsella and Abman, 2005). However, the unpleasant odor of H_2S may pose more problems for administration, which could necessitate the implementation of appropriate trapping systems to prevent from spreading into environment and exposure of medical personnel (Szabo, 2007). With enteral or parenteral formulations, odor would not create a problem, but manufacturing and formulation issues remains challenging compared with conventional chemical compound. In addition, animal safety data are still lacking with respect to parenteral or enteral administration of H_2S. Although sulfide is an endogenous substance, all exogenous sulfide delivery systems would be required to pass stringent safety and efficacy tests in preclinical animal studies before progression into human studies.

Of note, there are several limitations in the present study, namely:

1) The effects of NaHS may not entirely represent the physiological roles of H_2S in the tissues or cells since the formation of H_2S by the enzymes is significantly slower than that produced by NaHS dissolving in the water. However, the protective roles of NaHS in different cardiac disease models should not be

denied since different groups of scientist have confirmed the cardioprotective effect of NaHS or other H_2S-donors in various publications.

2) The determination of H_2S concentration in plasma or medium was performed by simple spectrophotomietric assay which involves some extreme conditions such as acidification of the samples. Although the absolute value of H_2S levels presented in the thesis is questionable, the relative changes of the H_2S levels in the samples after different treatments are still reliable.
3) The formation of HNO by H_2S+NO is not conclusively demonstrated because there is no specific and sensitive detection method for low amount of HNO generated in biological samples. More extensive chemical tests like high-performance liquid chromatography and X-ray crystallography may be used to determine the structure of the reaction product of H_2S+NO.

To further elaborate the significance of the present study, the future experiments should focus on several important areas:

1) Examine the effect of H_2S+NO in the whole heart or whole animal, to confirm its physiological or pathological relevance.
2) Confirm the chemical structure of the interaction product of H_2S and NO which may facilitate the discovery of its specific scavengers and its target proteins or receptors.
3) Improve the understanding of the physical, chemical and biological properties of this interaction product, including the life-span of this compound in the physiological environment.

Chapter 7 Conclusion

The current study demonstrated that H_2S negatively regulated β-adrenergic activation caused by ischemia/reperfusion stimulus. The cardioprotective effects were further proven by the findings of the involvement of endogenous H_2S in the protection conferred by either ischemic pre- or post-conditioning, and the cardioprotection of pharmacological pre- or post-conditioning with H_2S. The protective effects of H_2S may involve activation of several pathways including SarcK_{ATP} channel, PKC and Akt/eNOS. The present study illustrates the potential therapeutic role of H_2S in the treatment of ischemic heart diseases. However, although the cardioprotective effects of H_2S and NO have been consistently reproduced in vast amount of study, the clinical use of these gases should be applied with caution. This is because that the present desertation also demonstrated that H_2S and NO may interact with each other to produce a novel substance, which possesses opposite biological functions to those caused by either H_2S or NO alone. The discovery of this novel compound may be of importance in understanding the functional cross-talk between these two important gasotransmitters, and may potentially change the therapeutic strategies of treatment different cardiovascular diseases.

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